



Animal Models *in* Orthopaedic Research

edited by

Yuehuei H. An
Richard J. Friedman

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*To Kay Q. Kang, M.D.
Without her love, inspiration, and support,
this book would not have been possible.*

Yuehuei H. An, M.D.

*To my wife Vivian, and my daughters Arielle and Leah,
for their patience, understanding, love and support.*

Richard J. Friedman, M.D., FRCS(C)

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Preface

Research using animal models provides important knowledge of pathological conditions that eventually can lead to the development of more effective clinical treatment of diseases in both humans and animals. This book covers most of the major animal models used in studies of biomaterials and orthopaedic disorders. It is to be used as a reference book and is primarily directed towards surgeons, investigators, research fellows, graduate students, or anyone working in the field of orthopaedic or biomaterial research. It is intended to serve as a basis for a literature search before embarking on a detailed research project. It is possible that other scientists or physicians in areas unrelated to orthopaedic or biomaterial research may also find this material useful as a source of reference, or as a tool to expedite their research.

This book is an outgrowth of the editors' own quest for information about animal research methodology in orthopaedic and biomaterial research and, more importantly, represents tremendous support from the orthopaedic and biomaterial research communities. The 11 chapters written by the editors are a combination of knowledge gained from personal experience and the research literature. The remaining 20 chapters are contributions from 45 well-known experts in their fields of interest from throughout the world.

The book has 31 chapters and is divided into eight major parts. Part I is a general discussion about the care and use of laboratory animals and experimental designs in orthopaedic research. Part II describes the most commonly used evaluation methods in orthopaedic animal research. Detailed descriptions of common animal models used in orthopaedic research are given in Parts III–VIII.

The book is designed to be concise as well as inclusive and more practical than theoretical. The text is simple and straightforward. Appropriate numbers of tables, diagrams, line figures, and photographs are used to make the contents more vivid. The appendices include a list of periodicals and publications related to orthopaedic research, laboratory animals, and procurement sources. Full bibliographies at the end of each chapter guide readers to more detailed information on the subject. A book of this length cannot possibly discuss every animal model that has ever been produced in orthopaedic research, but it is felt that the major models and their applications have been included.

Yuehuei H. An
Richard J. Friedman

Foreword

Research is at the heart of progress in orthopaedic surgery as it is in all other fields of medicine. The products of research have changed the face of orthopaedics and have provided for millions of human beings the chance of enjoying active and productive lives.

Research encompasses a broad range of activities, but it should always begin with a burning question that will lead to the development of one or more hypotheses and the need to test them experimentally.

For that purpose, there are a number of methods available to the investigator. They include *in vitro* experiments, the use of cadaveric material, computer models, physical models, and clinical databases.

Animal models are an integral part of the process and, as such, are frequently used in orthopaedic research. However, prior to their application there are some fundamental issues that must be addressed. Is the animal experiment truly necessary or can the questions be answered using other methods?

Experimental work requiring the use of living creatures can never be taken lightly. An enormous controversy surrounds us regarding the ethical issues involved. Thus, the use of animal models for subjects that are important to mankind can only be justified when there is no other viable alternative.

Experiments in animals when justified and properly planned and executed have been essential in the acquisition of new knowledge.

Animal models have allowed us to understand the natural history of disease, to develop new and improved surgical techniques, and to predict the effect of a given treatment or surgical procedure. They have been critical in the development and in the evaluation of implants, one of the basic elements of modern orthopaedics. Animal models play a crucial role in biocompatibility evaluation, which is the most fundamental basis of knowledge necessary in the biomaterials field. Tissue engineering and the use of new technologies based on molecular biology developments require animal experiments. And the list can go on and on. In every aspect of orthopaedic research, the use of animal models constitutes an essential step that leads to the eventual application of newly acquired information to the human patient.

This work then addresses a very important subject and one that to my knowledge has not been covered in such a comprehensive manner in any other book or publication.

There are a number of features that make this book unique in addition to the subject and the depth in which it has been addressed. The editors have played a very active role in the conception and in the execution of the project. Of the 31 chapters that comprise the book, 11 were written by the editors themselves. This is unusual and reflects on the one hand their expertise and knowledge of the subject and, on the other, the level of their commitment.

The book is divided into eight parts. The first two deal with principles of detailed methodology. The other six address the use of models for specific purposes. Given the nature of the musculoskeletal system, a broad picture needs to be considered. Bone, cartilage, joint replacement, ligaments and tendons, spinal conditions and microsurgical techniques are included with chapters that address in detail the use of animal models in most areas of related research.

I particularly enjoyed Part One of the book, including all its five chapters, which by themselves represent in my judgment a major contribution. The ethical issues involved are presented in a very objective light, taking into account the concerns of the orthopaedic research community as well as those of animal rights advocates. This is a topic with which all investigators should be very familiar.

There is no research without appropriate experimental design. The young investigator will find important information to guide him from the conception of the basic ideas, to the development and execution of the experiment, and to the eventual publication of results in a scientific journal.

My own perception is that it will be among our young trainees and investigators that the book will have its major impact. The seasoned researcher and the basic scientist will find this book very valuable as well, and an excellent source of reference given the breadth with which the subject has been approached. This book has a great deal to offer to everyone involved in orthopaedic research.

Jorge O. Galante, M.D.

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Part I

General Considerations of Using Laboratory Animals



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1 Ethics and Regulations for the Care and Use of Laboratory Animals

*Alison C. Smith, Richard T. Fosse, Warren K. Ramp,
and M. Michael Swindle*

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I. INTRODUCTION

The use of animals has played a vital role in the numerous medical advances in the field of orthopaedic research. This is primarily because of the inherent limitations of alternatives to animal experimentation that preclude the study of the interactions of the various tissues and organ systems in the intact organism. A position statement issued by the American Academy of Orthopaedic Surgeons has endorsed the appropriate use of animals in orthopaedic research in addition to acknowledging the importance of increasingly refined alternative approaches. The group believes that the continued use of animals is justified based on the human and animal benefits to quality of life issues.¹ Other professional organizations engaged in biomedical research also have position statements regarding the use of animals.^{2,3}

The current climate for the conduct of biomedical research which involves animal use in general is stormy. Viewpoints are frequently polarized between the biomedical research community and animal protection groups. At their most extreme, these attitudes have resulted in the unfair characterization of

scientists as uncaring individuals who view animals as a means to an end and animal advocates as anti-intellectuals. The reality is that there are many caring scientists who approach the use of animals responsibly and many sensible animal advocates. In the end, research animals' best interests will be served when both groups find a middle ground from which a reasonable dialogue can ensue.

Although public attitudes have shifted towards support of animal welfare and animal rights viewpoints over the last 20 years, public opinion continues to strongly support animal experimentation. Recent polls indicate that 60–80% of the population accepts the use of animals in biomedical research; however, support for animal use varies with the species of animal used and the type of research.^{4,5} In general, the public is more concerned with the reduction and elimination of pain and suffering for research animals as opposed to experiments that require animals to be killed.

With that said, it is the intent of this chapter to briefly describe some of the ethical issues involved in animal research and to summarize the current regulatory standards under which biomedical research using animals, including orthopaedic research, is conducted. The regulations and guidelines for research animals are living documents and continue to be revised. Salient issues included in these documents involve minimizing animal pain and distress, the use of alternatives when appropriate, and justification of the number and species of animals selected.

II. ANIMAL RESEARCH AND ETHICS

A. IMPORTANCE OF ANIMAL RESEARCH

Public controversy regarding animal experimentation can be traced back two centuries with the establishment of the use of animal experimentation in scientific methodology. The antivivisection movement was established in response to the new field of experimental physiology, in which key discoveries were made possible by the use of animals.⁴ Many of the ethical issues born during this period are still intensely debated today. It is beyond the scope of this chapter to give a complete overview of the current major philosophical arguments regarding animal experimentation; however, the reader is referred to some of the many recent publications for further reading.^{4–12}

Defenders of animal experimentation generally argue in favor of the benefits of research to both humans and animals. Philosophically, this argument is in the utilitarian tradition wherein the consequences of a particular action are weighed in terms of benefit and harm to all those affected. The utilitarian or consequentialist approach suggests that the considerable benefits attained through animal experimentation easily outweigh the cost in terms of animal pain and distress.

Another approach in support of animal research is made by identifying distinguishing features that are morally significant and which separate man from animals. One of the most frequently debated issues is whether animals have moral agency. Carl Cohen of the University of Michigan, a supporter of animal research, argues that animals lack rights. He has written that rights are claims, or potential claims, that entail obligations among members of society. Since animals lack the ability to comprehend the rules that come with duties and do not have obligations, they have no rights.⁴

Philosophers have been some of the key voices and founding intellectuals in the modern animal movement. Peter Singer, the author of the seminal animal rights book, *Animal Liberation*, uses the utilitarian argument to attack animal research on the basis that moral status should be conferred on animals based on their ability to experience pain and suffering. Singer identifies the capacity to suffer with sentience and, therefore, with right to moral status. He argues that the benefits of animal experimentation are trivial compared to the cost of animal suffering. Singer does not confer moral equality between animals and man; instead he argues that equal consideration be given to animals when their interests (such as being hurt) are equal.⁵

The leading proponent of the position that animals should be accorded rights is philosopher Tom Regan. He asserts that animal life has inherent value which should accord animals the right to not be used as a means to an end. He believes that no matter how much good results, animal experimentation is morally wrong because it violates animals' basic rights.⁴

B. THE ‘THREE RS’ AND ALTERNATIVES TO ANIMALS IN ORTHOPAEDIC RESEARCH

The English scientists William Russell and Rex Burch, in their 1959 publication of *The Principles of Humane Experimental Technique*, were the first to voice scientists’ concern for experimental animals.¹³ They described the principles of the three “Rs” which promoted goals for research scientists: (1) replacement of animals by use of *in vitro* methods or by using animals that are phylogenetically lower; (2) reduction of the number of animals required; and (3) refinement of experimental methods to reduce the ethical costs in terms of painful or stressful procedures. Some decades later, the “three Rs” have been adopted by the scientific community and have served as the definition of the current search for alternatives.

In orthopaedic research, the state of the art is such that replacement alternatives are not developed to the point of eliminating all animal use. At this time, it is likely that most orthopaedic researchers would say that replacement alternatives will never be developed. However, all “three Rs” have current applications including the use of phylogenetically lower animals and the use of techniques such as cell/tissue cultures, benchtop experiments, and computer simulations. Cell and organ cultures can be used for toxicity and biocompatibility testing of drugs and biomaterials, as well as for evaluating effects of hormones, growth factors, and environmental factors on bone metabolism. This approach can give insight into direct effects of these influences on biological processes involved in bone diseases that would otherwise be difficult or impossible to obtain from whole animals. In addition, invertebrate animals and microorganisms are utilized for studies of mechanisms of biological mineralization and pathogenesis of osteomyelitis. Physical and computer models are also used and may be appropriate for studies of joint mechanics, prosthesis wear, and complex physiological systems such as calcium homeostasis, while cadaveric materials can be applied to development of surgical techniques and constructs. Computerized patient registries serve as excellent epidemiological databases for retrospective clinical studies and for outcome assessment. Compared to animal models, the advantages of these systems are that they are usually faster and less expensive, produce less pain and distress in vertebrate animals, and reduce the number of vertebrate animals used in research. Some alternative methods used in orthopaedic animal research are summarized in Table 1.

An issue that is likely to remain a source of controversy for orthopaedic researchers who use animals and for individuals who care for animals is whether surgical procedures of extremities should be performed unilaterally or bilaterally. From the scientific standpoint as well as in the interest of using fewer animals, the case is often made that a bilateral model is preferred since studies can be designed to allow an animal to be its own control. Unilateral models have the advantage of producing less pain and distress to the individual animal. Clearly, protocols of this kind must be carefully evaluated and the scientific merits weighed against the potential for pain and distress to the study animals.

III. LEGISLATION AND GUIDELINES

A. U.S. LEGISLATION AND GUIDELINES

1. In General

The conduct of biomedical research using animals carries with it the imperative that the care and use of animals is appropriate and their treatment is humane. In the United States, this imperative is in the form of two federal laws which govern the use of all animals used in research, testing, and education. The Animal Welfare Act (AWA) of 1966 (P.L. 89-544) and its subsequent amendments are administered by the United States Department of Agriculture (USDA) and implemented by USDA’s Animal and Plant Health Inspection Service (APHIS). The regulations which promulgate the AWA are published in Title 9 of the Code of Federal Regulations and are commonly referred to as the Animal Welfare Regulations.³⁶ The AWA regulates appropriate care and treatment for

TABLE 1
Examples of Alternative Testing Methods

Method	Material	Use ^{Ref.}
Cell cultures	Osteoblast	Osteopetrosis pathogenesis ¹⁴
		Poly-L-lactate biocompatibility ¹⁵
	Fibroblast, Myoblast	Particle toxicity ¹⁶
		Phenotypic expression ¹⁷
Organ cultures	Tibia	Titanium biocompatibility ¹⁸
	Tibia, Calvaria	Antibiotic toxicity ¹⁹
	Parietal bone	pH effects ²⁰
Bacteria	<i>Bacterionema matruchotti</i>	Estrogen effects ²¹
	<i>Staphylococcus aureus</i>	Calcification mechanism ²²
Invertebrates	Mollusk shell	Osteomyelitis pathogenesis ²³
	Crustacean shell	Mineral nucleation ²⁴
Cadaveric materials	Wrist	Calcification mechanism ²⁵
	Spine	Fracture fixation ²⁶
	Knee	Spine stabilization ²⁷
Computer models	Knee	Ligament reconstruction ²⁸
	Total hip prosthesis	Prosthesis development ²⁹
	Joint prosthesis	Implant positioning ³⁰
Physical models	Joint simulator	Wolff's law ³¹
	Joint loading model	Wear behavior ³²
Epidemiologic databases	Patient registry	Exercise physiology ³³
	Clinical and outcome data	Retrospective clinical studies ³⁴
		Testing treatment hypotheses ³⁵

animals used in research and includes provisions regarding their sale, shipping, purchase, housing, and veterinary care. The *Public Health Service Policy on Humane Care and Use of Laboratory Animals of 1986* (PHS Policy) was published by the Public Health Service (PHS) to implement The Health Research Extension Act of 1985.³⁷

In addition to the above laws, there are several federal documents that promulgate guidelines for institutions that receive federal funding. The PHS Policy endorses and supplements the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training*, a set of guidelines developed by the Interagency Research Animal Committee. The PHS Policy is applicable to all PHS-conducted or supported activities involving animals, regardless of the institution or country in which the work is conducted. The *NIH Guide for the Care and Use of Laboratory Animals* also endorses the U.S. government principles and its use by institutions as the basis for the development and implementation of animal care programs is required by the PHS. The guide contains guidelines concerning institutional policies and responsibilities; animal environment, housing, and management; veterinary medical care; and physical plant and is widely recognized as a primary reference on animal care.³⁸ These regulations will be summarized as they pertain to the field of orthopaedic research using animals, specifically issues concerning surgery and minimizing pain and distress.

2. Definitions

Part 1 of the AWA defines terms used in parts 2 and 3, which outline the law's requirements. While the guide lacks a list of definitions, it does define certain issues related to surgery in the body of the text. Both the USDA regulations and the NIH guide define a major operative procedure as any surgical procedure that penetrates and exposes a body cavity or produces permanent impairment

of physical or physiological functions. Both the USDA regulations and the guide prohibit multiple major survival surgery on a single animal unless it has been scientifically justified by the principal investigator and approved by the Institutional Animal Care and Use Committee (IACUC) or unless it is part of routine veterinary care required to protect the health of the animal.

From a veterinary care and animal welfare perspective, a prevailing issue is the ability to conduct animal research while minimizing pain and distress. The USDA regulations define a painful procedure as any procedure that could reasonably be expected to cause more than momentary or slight pain or distress in humans in excess of that of an injection. Although neither the guide nor the PHS policy include such a definition, the fourth Government Principle states that procedures that cause pain or distress in humans should be considered to cause pain or distress in animals.

Another term that is defined by the USDA regulations and that is relevant to the issue of pain and distress is that of a paralytic drug. The USDA defines a paralytic drug as one which produces partial or complete loss of muscle contraction and which has no anesthetic or analgesic properties, so that the animal cannot move, but is completely aware of its surroundings and can feel pain. The guide specifically states that paralytic drugs are not anesthetic or analgesic.

3. Institutional Policies and Responsibilities

The USDA regulations, the guide, and the PHS policy call for the appointment by the chief executive officer at each research institution of an Institutional Animal Care and Use Committee (IACUC). IACUC members must be qualified through experience and expertise to oversee and evaluate the institution's animal program, procedures, and facilities. The composition of the IACUC varies depending upon which of the regulatory documents are cited, but all call for membership to include a doctor of veterinary medicine with training or experience in laboratory animal science and medicine and one public member not affiliated with the institution in any way other than as a member of the IACUC. The PHS policy and the USDA regulations both state that the veterinary member have direct or delegated program authority and responsibility for activities involving animals at the research facility. The PHS policy and the guide state that one member should be a practicing scientist experienced in research involving animals.

Common to all three regulatory documents are the following IACUC functions. The IACUC must conduct a complete review of an institution's program of animal care and animal facilities every six months. It then must prepare and submit reports of its evaluations to the institutional official. The committee is also charged with the review of all protocols that involve the use of animals. The committee has the authority to review and approve, require modifications to secure approval, or withhold approval of proposed procedures involving animals and proposed significant changes in ongoing protocols.

4. Protocol Review

Review and approval of the animal use components of a research proposal is one of the principal charges of the IACUC, and approval must be obtained before beginning any portions of the research project that involve animal use. The regulations differ somewhat regarding some specific aspects of protocol review. The USDA regulations require identification of the species and approximate number of animals requested. The USDA regulations and the guide require that the principal investigator provide a rationale for using animals and justification for the number of animals to be used. The guide further specifies that the number of animals requested should be justified statistically whenever possible. The third U.S. government principle makes a broader statement regarding the justification of animal use by stating that animals should be selected "of an appropriate species and quality and the minimum number required to obtain valid results." The USDA regulations require a complete description of the proposed use of the animals and the procedures that will be used to limit discomfort and pain to that which is unavoidable to conduct the study.

The USDA regulations and the PHS policy state that procedures which involve animals should be designed to avoid or minimize discomfort, pain, and distress. The USDA regulations further specify that a principal investigator must have considered alternatives to procedures that may cause more than momentary pain or distress and that documentation be provided regarding the methods used to determine that alternatives were unavailable. Consistent with the idea of reducing and replacing the use of animals, both the guide and the third U.S. Government Principle recommend the use of *in vitro* biological systems or computer simulation when appropriate. Unnecessary duplication of previous experiments is discouraged by both the guide and the USDA regulations, and a written assurance to that effect is required by the USDA regulations.

All the regulatory documents require the appropriate use of sedatives, analgesics, or anesthetics for procedures that may cause more than momentary or slight pain or distress unless the withholding is justified for scientific reasons in writing by the investigator. In addition, they also use similar wording to address the issue of severe or chronic pain or distress that cannot be relieved by stating that those animals should be euthanized at the end of the procedure or, if appropriate, during the procedure. The USDA regulations require veterinary consultation during protocol planning for any procedures that may cause more than momentary or slight pain or distress.

Use of paralytic drugs without anesthesia for surgical or other painful procedures is absolutely contraindicated by the guide, the USDA regulations, and U.S. Government Principles. Because they lack either analgesic or anesthetic properties, the concern is that animals which are immobilized but still conscious could experience pain or distress. The guide makes specific recommendations regarding their usage when combined with anesthetics for surgical procedures. The guide also recognizes that their use as a sole agent may be appropriate for certain nonpainful, well-controlled studies. However, it also definitively states that any proposed activities of this sort receive a careful evaluation by the IACUC to ensure that animal well-being is safeguarded.

All the regulatory documents use similar wording regarding qualifications of personnel conducting procedures on animals. The USDA regulations, the guide, and the PHS policy specifically charge the committee to evaluate and determine that personnel conducting procedures on animals have received adequate training in the procedures used. Assessment of personnel qualifications is required regardless of professional degree since it is implicit in the requirements that specific knowledge regarding animal anesthesia, surgery, or other experimental techniques is necessary for performing these tasks in a humane and scientifically acceptable manner.

Provision of adequate veterinary care is a requirement in all the regulations and guidelines. The USDA regulations and the guide also require appropriate provision of pre- and post-operative care for animals undergoing surgery. The guide makes specific recommendations regarding preoperative planning, monitoring during surgery, and provision of post-operative care.

The USDA regulations and the guide address issues specific to protocols which involve surgery. The USDA regulations require that all survival surgery be performed using aseptic procedures and techniques. They further specify that major operative procedures on non-rodent species may be performed only in dedicated facilities maintained under aseptic conditions. Minor surgical procedures and survival surgery on rodents do not require dedicated facilities, but must be performed using aseptic procedures. The guide makes similar recommendations, but recognizes that modifications to aseptic procedures may be appropriate for certain surgical procedures in rodents. The USDA regulations describe aseptic procedures to include the use of surgical gloves, masks, sterile instruments, and aseptic techniques. The guide gives a similar description of aseptic technique but elaborates specific practices involved in its application.

Whereas the USDA regulations specify only that survival surgery on nonrodent species be performed in a dedicated facility maintained and operated using aseptic procedures, the guide provides more specific recommendations on facility design. The guide divides the functional components of an aseptic surgery suite into surgical support, animal preparation, surgeon's scrub, operating room, and post-operative recovery. Design features that help minimize traffic flow and contamination are recommended.

Despite the fact that more than one set of regulations governs the use of animals in orthopaedic research, there is general accord among the pertinent laws and guidelines. The implementation of the regulations will be unique to each research institution based on its available facilities and research program. Legal authority for upholding the applicable regulations rests with the IACUC; its responsibilities for oversight of an animal care program are numerous and specific. Ultimately, a successful animal care program is based on a marriage of the professional standards described by the regulations and their implementation using professional judgment.

B. EUROPEAN LEGISLATION

1. In General

Countries in Europe, led by the United Kingdom, have a long history of legislative regulation of biomedical research with animals. The British Anti-Cruelty Act was introduced by Richard Martin in 1822. He later founded the Royal Society for the Prevention of Cruelty to Animals. The British Cruelty to Animals Act regulating animal experimentation was introduced in 1876. Animal protection in France can be traced to the Grammont law of 1850. France passed legislation specifically applied to animal experimentation in 1963. Similar laws have been passed in most European countries.

Europe has been in the process of harmonizing several of the regulations that cover many activities, including experimentation with animals. Central to European legislation are the “three Rs” proposed by Russell and Burch.¹³ The concepts of reduction, replacement and refinement are the basic concepts that have formed the foundation of the main sets of European laws and regulations.

Currently in Europe there are two collaborative blocs that have led to common legislation regulating the use of animals in biomedical research. Experimentation using animals is regulated in Europe by two sets of transnational laws and treaties, the European Union Directive 88/609,³⁹ and the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimentation and other Scientific Purposes, CoE ETS 123.⁴⁰

2. European Directive 88/609

The European Union (EU) comprises 15 member states that have agreed to cooperate in a number of areas that primarily regulate free flow of goods and economic activity in the member state areas. The process — commonly called harmonization — is designed to establish a free European economic zone with a common set of minimal laws.

The EU adopts legislation that has different levels of application. Regulations serve as binding laws in the member countries. Directives set minimum requirements designed to achieve a desired result but allow some degree of discretion that can be exercised by each member state; decisions are binding only to certain parties. The directive 88/609 is an example of legislation that allows some degree of discretion that can be exercised by each member state. Common to all the member states’ legislation is a minimum requirement that serves to coordinate legislation.

3. CoE Convention ETS 123

The Council of Europe (CoE) is an international organization currently comprising 40 member states, including the 15 member states of the European Union. Treaties and conventions adopted by the CoE members are not laws that are applicable in all the member states. The decisions of the CoE become binding only after a certain number of member states ratify a convention. The CoE convention became binding after three states ratified the convention. Unlike the EU which has an economic base for its legislative focus, the CoE is concerned with issues of democracy, human rights and the adoption of common practices, in this case common minimum rules for the use of animals in biomedical research.

The two sets of laws are very similar and for the purposes of this chapter can be viewed as being identical. Therefore, the CoE convention will be used as the legislative system to describe how biological experiments with animals in the majority of European states are regulated. The convention opened for signature on March 18, 1986, and had as its aim the protection of vertebrate animals used for experimental and other scientific purposes. The convention has defined sets of criteria for the treatment of animals that apply in all the 40 member states. These cover guidelines for housing, breeding, sources of animals, and the competence of persons who have responsibility for care of animals, or the performance of procedures, whether these be planning or practical hands-on procedures. The convention also allows for the introduction of quality standards. It is reviewed at multilateral negotiations held in Strasbourg every third year. These meetings are designed to review the application of the convention and suggest modifications that may be needed at any time.

The most significant area that has been addressed is that of competence. The convention states that all persons engaged in research with animals shall be deemed competent (Article 26 CoE and Article 14 EU). No person in the CoE/EU area shall be allowed to perform or plan procedures involving animals, or assume responsibility for planning procedures on animals, unless he/she has taken part in training specified by the CoE/EU. The implication of this is that uniform competence requirements are in the process of being adopted in all the participating countries. The EU established an expert committee under the auspices of the Federation of European Laboratory Animal Science Association with a mandate to suggest curricula for training and teaching personnel in Europe.⁴¹ The committee defined four categories of personnel: caretakers (Group A), laboratory animal technicians (Group B), researchers (Group C), and laboratory animal specialists (Group D). Similar recommendations have been proposed for euthanasia,^{42,43} and microbiologic quality.⁴⁴ The convention states that stray or ownerless animals shall not be used, and that all cats and dogs shall be purposely bred. The same applies to all other species with the exception of farm animals and wild caught primates. Minimum requirements for housing, cage sizes, and for environmental enrichment are specified, but it is up to each member country to exceed these specifications.

The convention does not require the formation of institutional animal care committees at each user establishment. Some form of peer review authority is required, but this is left to the discretion of the member country. This has led to the development of several systems ranging from local ethical committees in several countries, e.g., Sweden, to decentralized responsible persons who act as extended forms of a central departmental level, e.g., Norway. Other countries have instituted systems of inspectors who certify the use of animals and monitor the procedures and institutions, e.g., the U.K., the Netherlands. There are varying degrees of lay person involvement. All countries require that the researcher obtain written permission prior to performing an experiment. Permission is granted following review by an official authority, and on condition that the applicant is competent to perform the procedures that are specified in the application. Each application shall demonstrate that the procedure cannot be performed without the use of animals, and that a suitable animal-free alternative is not available.

C. LEGISLATION AND GUIDELINES OF OTHER COUNTRIES

To the authors' knowledge, similar legislation, guidelines, and policies on the care and use of laboratory animals exist in several other countries, reflecting a worldwide concern for animal welfare. These countries include Canada, Australia, Japan, and Taiwan. In Canada, federal legislation which governs animal welfare is under Section 446 of the Criminal Code. In addition, numerous provincial acts pertain specifically to the use of experimental animals in research, teaching and testing. The *Guide to the Care and Use of Experimental Animals* contains guidelines established by the Canadian Council on Animal Care to evaluate animal care and use in Canadian universities and government and commercial laboratories.⁴⁵ The *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* and its subsequent revisions cover all live vertebrate species used for scientific purposes in research, agriculture, biology, industry, and teaching. The code

describes the responsibilities of institutions and individuals that use animals as well as requirements for their procurement and care.⁴⁶ In Japan, research using animals is closely regulated by the government under the *Standards Relating to the Care and Management of Experimental Animals*, which is part of the Law Concerning the Protection and Control of Animals. The Japanese regulations are comprehensive and include policies related to animal transport, animal health, public health, waste disposal, and breeding.⁴⁷ Researchers in the above-mentioned countries or other countries should observe the national or local legislation, regulations, or guidelines on the care and use of laboratory animals and help to promote them.

IV. OBTAINING APPROVAL FROM THE IACUC

In order to fulfill its responsibilities concerning protocol review, each IACUC at a research institution requires the submission by the principal investigator of a written protocol which completely describes all aspects of animal use involved in a research protocol. The protocol must address the specific federal requirements described above as well as any applicable state, local or institutional requirements. If an institution receives federal funding or is accredited by AAALAC International, it is compelled to meet the requirements described in the PHS Policy and the guide as well as to comply with the legally binding standards in the USDA regulations. This is the case for the majority of the institutions in the United States which utilize animals to conduct orthopaedic research.

The actual format of the form used by the IACUC for protocol review is generally individualized by each institution. This also applies to the way in which the principal investigator is asked to provide the relevant information. For example, some forms will ask a series of very specific questions, with additional elaboration prompted by response to a particular question or section. Other formats may be less structured and simply ask for a narrative description to questions or information requested. Regardless of the format for animal protocol review used by a particular IACUC, the principal investigator should be aware that the information which she/he is requested to provide is based on the current applicable regulations and guidelines. Responses should be concise but not so abbreviated as to omit relevant and required information, e.g., dosages for drugs, their routes of administration, and dosing schedule.

The perception that more “paperwork” is now necessary for approval of animal protocols compared to past requirements is widespread and is actually true to some extent. This is due to the fact that revisions continue to be made in the pertinent regulations and guidelines. Impetus for change has come from both public concern regarding the treatment of animals used in biomedical research as well as from the scientific community’s commitment to animal welfare and the need to formulate standards in step with current scientific knowledge and technology. It should be remembered, however, that responsibility for the ethical and humane use of animals used in research begins with the investigator’s decision to use animals. The required process of protocol submission, review, with appropriate modifications when necessary, and approval is the mechanism by which investigators can best justify and describe the necessity to use animals. By providing appropriate responses to the information required, the investigator not only expedites the entire review process, but also satisfies the societal demands for accountability that are represented by the applicable regulations and guidelines.

V. GOOD LABORATORY PRACTICE

Products such as medical devices intended for human use, human and animal drugs, and biological products are regulated by the Food and Drug Administration (FDA) and must undergo preclinical testing prior to clinical testing. Standards exist for testing such FDA regulated products that support applications for research or marketing permits in the document entitled *Good Laboratory Practice (GLP) for Nonclinical Laboratory Studies*.⁴⁸ Adherence to the practices described in the document

is intended to assure the quality and integrity of the safety data filed in accordance with applicable sections of the Food, Drug, and Cosmetic Act and the Public Health Service Act. The regulations concerning GLP studies are comprehensive and include standards for all personnel, facilities, equipment, test articles, and records involved in a nonclinical laboratory study.

Any facility which conducts a GLP study is subject to inspection by the FDA. The FDA must be permitted access to inspect the facility and any records or specimens maintained as part of a study. Copying of records must also be permitted. Any facility which does not permit inspection by the FDA will not have its nonclinical laboratory study considered by the FDA for support of an application for research or marketing permit.

All personnel involved in conducting a GLP study must have the qualifications to perform their assigned functions. Qualifications can include education, training, and experience or a combination thereof, and must be documented for all individuals involved. A study director with the appropriate professional background must be designated for each GLP study and has overall responsibility for the technical conduct of the study as well as the interpretation and reporting of results.

A testing facility must have a quality assurance unit which is independent of the personnel who conduct the study. The quality assurance unit is responsible for monitoring each study to provide assurance that the facilities, equipment, personnel, methods, practices, records, and controls conform with the regulations. The quality assurance unit is charged with performing and documenting periodic inspections of a study. Any problems encountered during the inspection must be immediately identified to the study director and management.

The regulations contain specifications regarding animal facilities, support areas, and laboratory space. Animal facility design must assure separation of species or test systems, separate individual projects, provide for quarantine of animals, and accommodate routine or specialized housing. In addition, there must be sufficient space to archive all raw data and specimens from completed studies with access limited to authorized personnel.

Equipment used during a GLP study to collect and assess data must be adequately tested and calibrated. Documentation of these procedures is required, as written provisions in the event of equipment malfunction.

Standard operating procedures (SOPs) must be in place to assure the quality and integrity of the data collected during the course of a study, and any deviation from the written protocol must be documented as part of the raw data. SOPs should include at a minimum animal care procedures; animal facility procedures; methods used to receive, identify, store, handle, mix, and sample control and test articles; animal observations; laboratory tests; procedures for handling animals found moribund or dead during a study; necropsy procedures, collection and identification of specimens; histopathology; data handling, storage, and retrieval; maintenance and calibration of equipment; and animal transfer, placement, and identification. Special emphasis is given to having procedures in place that could affect the outcome of the study, such as inadvertent animal misidentification or exposures to test or control articles. Food and water provided to animals must be analyzed periodically during the course of the study for contaminants that might reasonably be expected to be present and affect study results.

The entire GLP study must be conducted in written accordance with the study protocol, appropriately documented by the individuals involved, and all deviations recorded. At the conclusion of a study, a comprehensive final report, signed by the study director, must be prepared that includes the objectives of the study, all methodologies involved in data collection and analysis, a description of the animals used, identification of all personnel involved in the study, and a description of where all raw data and specimens will be stored.

Finally, the regulations set forth conditions under which testing facilities may be disqualified for failing to comply with the requirements. If a facility has been disqualified, it can seek to be reinstated by providing evidence to the Commissioner of the FDA that it has taken appropriate corrective actions to assure compliance with the regulations.

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2 Experimental Design, Evaluation Methods, Data Analysis, Publication, and Research Ethics

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I. QUESTION, HYPOTHESIS AND EXPERIMENTAL PURPOSE

The first step in generating a research project is the formulation of a new idea or question to be investigated. Orthopaedic surgeons often formulate research ideas when they encounter problems during their routine diagnostic, treatment and follow-up procedures. Ph.D. researchers or others who work exclusively in the laboratory may derive research ideas from conversations with orthopaedic surgeons or by reading the biomedical literature. It is often difficult to determine the most appropriate manner in which to investigate the idea. Although surgeons are the original source of many research ideas, researchers are usually more informed on the biomedical literature. The literature is one of the most important tools in helping researchers to evaluate the originality and feasibility of testing a particular idea. Occasionally, what had seemed to be a “new idea” to a surgeon may already have been tested and published in the literature. So, whether the primary investigator is a surgeon or a laboratory researcher, a thorough search of the literature is necessary in order to assess the originality, necessity, and the feasibility of carrying out the project. A review of previous studies allows the researcher to refine and reformulate the hypotheses, designs, methods and tools employed previously. The new internet technology has made medical and research literature much more accessible. However, one must still go to a library and obtain the original papers because abstracts are too brief to give all of the necessary information.

The hypothesis is an unproven theory which is tentatively put forward in order to be tested. It is a more specific, logical and scientific form of a thought or idea and consists of the main question or questions to be investigated. Several hypotheses may be involved in the verification of one idea.

The experimental purpose is a statement of the general goal of the study. It states exactly what the investigator plans to accomplish and it must be based on established experimental designs and evaluation methods. It is impossible to conduct a good, relevant research project without a specific question, clear hypothesis, or a well-defined purpose.

The following examples may help readers understand the meanings of and the relationships between the idea or question, the hypothesis, and the experimental purpose. Example 3 also demonstrates the logical progression of experimental projects which may arise from a single idea.

Example 1 Mechanical Symmetry of Rabbit Bones¹

a. Brief Review and Questions Bilateral animal models are used extensively for evaluating biocompatibility and biofunctionality of biomaterials in bones. Implicit in such an approach is the assumption that, in the same animal, a pair of corresponding right and left bones have similar mechanical properties. Only a few published articles, however, verify this assumption. The rabbit is commonly used in orthopaedic animal research and the mechanical properties of rabbit bones

TABLE 1
Comparison of Long Bone Mechanical Properties between
Normal Control, Contralateral Control, and Treatment
Groups*

Bone	Treatment	Side	Strength (MPa)	Elastic modulus (GPa)
Femur	Normal control	Both femur	130 ± 5	13.6 ± 0.4
	Arthritis model	Left (control)	97 ± 21	8.3 ± 1.5
		Right (arthritis)	80 ± 16	7.1 ± 1.4
Tibia	Normal control	Both tibia	195 ± 6	21.3 ± 0.7
	Arthritis model	Left (control)	186 ± 26	15.9 ± 2.9
		Right (arthritis)	173 ± 19	14.9 ± 2.2
Humerus	Normal control	Both humerus	167 ± 5	13.3 ± 0.6
	Arthritis model	Left (control)	158 ± 25	11.9 ± 2.7
		Right (arthritis)	157 ± 26	12.4 ± 2.4

* The values were generated using a three-point bending test.^{2,3}

are important parameters in many experimental designs. Thus, studies are needed to evaluate the mechanical symmetry of rabbit long bones. This will allow better experimental designs to be created.

b. Hypothesis and Purpose The hypothesis of this study is that the mechanical properties of rabbit long bones are symmetrical. The experimental purpose of the study is to verify the hypothesis by examining the bending and indentation parameters of rabbit long bones on each side. The results demonstrated no significant differences between the right and left femur, tibia, or humerus for any of the bending or indentation parameters (Table 1).

Example 2 Bone Ingrowth to Implant Surfaces in Osteopenic Bone⁴

a. Brief Review and Questions Total joint replacements are a mainstay of treatment for debilitating arthritis. Many of these procedures are performed on patients with osteopenic bone due to inflammatory arthritis or osteoporotic bone conditions. Further research is needed to determine whether the osteopenia has detrimental effects on bone ingrowth into the implant surfaces (Question 1). It is also known that under normal conditions bone ingrowth varies with different implant surfaces (this includes the material composition of the implant and the surface texture of the implant). Is this also true in osteopenic bone (Question 2)? Intra-articular injection of carrageenan has been shown, in previous studies, to induce an inflammatory arthropathy and peri-articular osteopenia in rabbits (Review for method selection). This provides a useful and appropriate model for the investigation.

b. Hypothesis and Purpose The hypotheses of this study are that bone ingrowth into an implant surface is different in osteopenic bone (Hypothesis 1) and that the ingrowth of osteopenic bone is altered by different implant surfaces (Hypothesis 2). The experimental purpose of this research project is to use a carrageenan-induced arthritis model and three different implant surface textures to evaluate the bone-implant interface and surrounding cancellous bone in the distal femur of adult rabbits. The contralateral femurs will serve as the treatment controls. They will have identical implants placed, but will not receive carrageenan injections. Both histomorphometric analysis and mechanical testing will be performed to determine the quality and quantity of bone ingrowth, as well as the mechanical strength of the interface. The results demonstrated that carrageenan-induced arthritis influences the quality of the adjacent bone and subsequently affects the bone ingrowth into different implant surfaces.

Example 3 Effects of Albumin Coating on Bacterial Adhesion and Implant Site Infection Rate

a. Study 1⁵ One of the most devastating complications in the surgical treatment of arthritis is infection. It may lead to complete failure of a joint replacement, occasionally necessitating amputation of the extremity. It has been demonstrated that adhesion of bacteria to a biomaterial surface is the initial step in the development of prosthetic infection. This adhesion may occur during the surgical procedure (through the air or by direct contact) or after implantation (by direct seeding from the bloodstream). It is also known that serum proteins, such as albumin, have inhibitory effects on bacterial adhesion to biomaterial surfaces.

A hypothesis is formulated to address the question of whether or not albumin-coating will effectively inhibit bacterial adhesion to implant surfaces (Question 1). The hypothesis is that albumin coating will reduce the number of *Staphylococcus epidermidis* which adhere to a titanium surface (Hypothesis 1). The experimental purpose is to use a computerized epifluorescent bacterial counting method to quantify the number of bacteria adhering to albumin-coated and non-albumin coated titanium surfaces, *in vitro* (Purpose 1). The results demonstrated that serum albumin coating inhibits *S. epidermidis* adhesion to titanium surfaces by more than 95 percent.

b. Study 2² After demonstrating the inhibitory effects of albumin coating with respect to bacterial adhesion, a subsequent question arose. How long would the albumin coating persist under physiologic conditions (Question 2)? Titanium implant surfaces were coated with bovine serum albumin (BSA) using a cross-linking agent. The implants were then placed in a solution of phosphate buffered saline at 37°C with agitation to simulate a physiologic environment. The inhibitory effect on bacterial adhesion was then evaluated at different time periods. The results revealed that only 10% of the coated bovine serum albumin (BSA) decayed off the surface during a 20-day incubation period and the inhibitory effect of the albumin coating on bacterial adherence remained high (more than 85 percent) throughout the length of the experiment (20 days).

c. Study 3⁶ The knowledge accumulated in the above studies established the potential effectiveness of albumin coating in preventing prosthetic infection. However, these were *in vitro* experiments. Could the results be replicated *in vivo* (Question 3)? The new hypothesis is that the cross-linked albumin coating will effectively reduce the frequency of prosthetic infection *in vivo* (Hypothesis 3). The purpose of this study is to use a rabbit model to evaluate the *in vivo* effects of a serum protein coating on the implant infection. The results of the experiment demonstrated a much lower infection rate (27%) in rabbits with albumin coated implants vs. those with uncoated implants (62%).

II. WRITING A RESEARCH PROPOSAL

The research proposal is a document which reflects the extensive investigation into the validity and originality of a particular idea and describes exactly how the experiment will be carried out. It normally consists of background information on the subject, the specific aims of the study, an experimental plan, the materials and methods which will be used, the significance of the project, and appropriate references.

The research proposal is essential for the following reasons: (1) it facilitates the formulation of a detailed experimental protocol which precisely defines the hypothesis, the purpose, the experimental plan, the evaluation methods, the potential difficulties and possible solutions, the expected results and the appropriate statistical methods for analyzing the data; (2) it provides written documentation that the idea is original and viable by referencing related work already completed on the subject; (3) it provides a tool for communicating with other researchers in the field, thus allowing further verification of the idea and possibly eliciting useful suggestions before the project is started; (4) it provides a detailed outline of the project, which serves to motivate all personnel involved, defining the role of each individual and the exact manner in which the goals of the project will be accomplished; and finally, (5) with certain modifications, it may serve as a grant application for gaining financial support from the local institution or other research foundations.

Details on how to plan a new project and how to write a research proposal can be found in the books by Ingle,⁷ Leedy,⁸ Hawkins and Sorgi,⁹ and Manly,¹⁰ or the review by Mendenhall.¹¹ Several important aspects in the development of a research proposal in orthopaedic animal research will be discussed, including determination of the number of animals (sample size) to be used, randomized sampling, designing control groups, and common experimental designs based on statistical principles.

III. EXPERIMENTAL DESIGN

A. NUMBER OF ANIMALS REQUIRED (SAMPLE SIZE)

The number of animals needed for a particular study can be determined based on information acquired from the literature or from the results of preliminary studies. The required number depends on the intrinsic variability among the animals being used, the consistency of the surgical procedure which will be performed, the accuracy of the evaluation methods, and the statistical techniques which will be used to analyze the data.

Statistically, sample size is related to power, effect size, and significance level.¹² It is important to realize that sample size calculations will always be approximate and that it is impossible to predict the exact outcome of any particular experiment.¹³ Many competent researchers lack sufficient statistical training to determine the appropriate sample size, the validity of statistical principles employed in an experimental design, and the statistical methods for data analysis. This is particularly true when sophisticated analysis is required. Consultation with a trained statistician is strongly advised.

1. Review of Similar Previous Studies

It is very helpful if information from previous studies with similar designs is available to the researcher. According to the standard deviation or coefficient of variance, the required number of animals can easily be estimated. For example, the number of rats (precise specimens) needed to study fracture healing for one time period using one evaluation method (histomorphometry or mechanical pushout test) is 8–12; the number of rabbits (precise specimens) required to study bone ingrowth in a femoral condyle using one evaluation model is 8–10; and the number of rabbits needed for the evaluation of different infection rates in two or more implants is 10–15.^{6,14}

These numbers may seem empirical. However, based on the belief that the majority of materials in the literature are statistically sound, one can assume that the numbers adapted from the literature were obtained by precise statistical analysis. A statistician should be consulted, however, to verify the accuracy of the estimation before the experiment is started or before a manuscript is submitted for publication.

2. Preliminary Data

If a suitable number cannot be determined after consulting other investigators and extensively searching the literature, a separate preliminary study should be designed. This pilot study should be designed such that only a few animals or specimens are evaluated. Usually one or two standardized evaluation methods may be used to determine the appropriate number of animals to be used in the project. For example, 6–8 animals or specimens can be used in a preliminary study with histomorphometrical analysis or mechanical testing as evaluation methods. Based on this preliminary study, the standard deviation (SD) of the mean (of a specified parameter, such as bone density, bone mechanical strength, or cartilage thickness), coefficient of variation, and mean difference among groups (effect size) can be determined.

Sample size is one parameter which helps to determine the power, effect size (or magnitude of the effect) and level of significance of a study.¹² The power of a study is the likelihood of rejecting

the null hypothesis. An 80% level is generally viewed as adequate. Effect size is a measure of the difference among the groups. Cohen¹⁵ defines a small effect as 0.2 of a standard deviation, a moderate effect as 0.5 of a SD, and a large effect as 0.8. It is more difficult to detect a small effect of the independent variable than it is to detect a large effect. So, if a small difference is expected between the control and treatment group, a relatively large sample size is necessary. The significance level is the probability of rejecting a true null hypothesis; it is often set at 0.05. In the book by Cohen,¹⁵ both power and sample size tables can be found. When planning a study, the researcher should determine the desired power, acceptable significance level, and expected effect size and use these three parameters to determine the necessary sample size.¹²

3. Animal Variance

In order to reduce the inter-animal variance and thus limit the number of animals needed, the study group should be as homogenous as possible. Ideally, the animals should be of the same strain, sex, age, weight and similar serum antibody status if applicable. Several experimental animal strains can be obtained which, due to extensive inbreeding, have very similar or identical genetic makeups. This is the case for SD rats, NZW rabbits and some strains of minipigs. Dogs and cats, on the other hand, are relatively heterogeneous, with respect to strain, age and body weight. Therefore, in a similarly designed study employing cats and dogs, the required number will be larger than with the other animals.

Two strategies which can markedly reduce the total number of animals needed are the use of paired designs and the use of multiple specimens in a single animal. In a paired design, the contralateral limb in each animal acts as the control. For example, four pairs of cortical plugs (each pair consisting of an experimental plug and a control plug) can be implanted into the femurs of a single animal. Each of the experimental plugs can be compared to a control plug which was implanted in a similar location in the contralateral femur. This arrangement provides nearly identical environments for the experimental and control groups which increases the power of the study. Note that, when using a cortical plug model, unicortical implantation is recommended. The reasons for this will be discussed later in the chapter. If two implant surfaces need to be compared for one time period, four dogs are normally enough for histological analysis (two animals) and mechanical testing (two animals, pushout test in most cases). According to previous studies, 6–8 pairs of specimens are enough for histomorphometrical evaluation or mechanical testing (pushout test) using paired student t-test for data analysis.

4. Evaluation Methods

The number of animals needed also depends on the design of the experiment, the evaluation methods and the statistical test for data analysis. For qualitative methods, such as descriptive histology, 2–4 animals (or specimens) are enough. For quantitative analyses, such as histomorphometrical or biomechanical evaluations, analyzed with a paired student t-test or repeated measures ANOVA, the number of animals (or specimens) in each group should be at least 6–8. Analysis of data with an unpaired student t-test normally requires a sample size of 8–12. Cohen's book¹⁵ provides tables that may be used to help to determine the appropriate sample size for a particular statistical test.

B. RANDOMIZATION AND SAMPLING ERRORS

Randomization involves dividing a population into two or more groups such that every individual has an equal chance of being placed into each group. Unless study subjects are completely homogenous, they should be randomized. This is essential before a valid conclusion can be drawn about a causal relationship between a treatment and the observed effect. This strategy attempts to equally distribute characteristics across experimental groups and thereby eliminate selection bias.¹⁶

Randomization of animals or specimens into different groups can be performed by using a random permutation table, throwing dice, drawing in a lottery or by using computer-generated random permutations. The latter is presented in detail by Martin et al.¹⁷

The random assignment of subjects to groups does not guarantee the equivalent distribution of all extraneous variables in the group. There must be a sufficient number of animals or specimens in each group for randomization to have a high probability of distributing the extraneous variables similarly across all groups. The distribution of identifiable variables, such as weight, age and sex should be evaluated after randomization is complete to determine whether they have been equally distributed.

When using multifactorial designs (e.g., three treatment groups and three time periods) or designs requiring a large number of animals, animal facilities may not be capable of housing all the animals at the same time. Thus the animals will need to be delivered on two or more occasions. All animals need to be randomized for study participation, but this situation is complicated by the potential differences among the animals delivered at different times (i.e., age, body weight, or susceptibility to disease). To eliminate this source of variation, all treatments should be equally represented in the animals from each delivery.

The basic objective of sampling is that a sample should be chosen to represent its population. An estimate of a population parameter that is determined from a random sample will generally differ from the true value to some extent. This difference is referred to as the sampling error. Sampling error reflects the inherent uncertainty of conclusions about a population based solely on information gained from sample data (a subset of the population). The magnitude of sampling error is a function of sample size. This is due to the large amount of inherent variation in estimates based on small samples as compared to the smaller inherent variation seen with large sample sizes. The amount of statistical uncertainty associated with a particular study can be expressed in the form of confidence intervals. These intervals are largely determined by the sample size. Intervals based on small samples are relatively wide, reflecting a relatively large sampling error. Intervals based on adequate sample sizes are more narrow, reflecting a smaller sampling error.

Two sampling procedures which are commonly used in biomedical research are simple random sampling and stratified sampling. A simple random sample is one that is drawn from a population (a larger animal group or tissue specimen) such that each element of the population has an equal probability of being included in the sample.^{10,18} Theoretically, it allows the knowledge gained by close examination of the sample group to be extrapolated to the larger population. Simple random sampling is useful in many situations; however, it does have some disadvantages. One problem encountered with simple random sampling is that, by design, there is no control over how the sample items are distributed in the population. This becomes an issue in cases where it is necessary to have the sample items spread out evenly within the population. For example, when viewing histologic sections of a tissue with a heterogeneous or anisotropic structure (such as cancellous bone), it is important to view sample sections which are distributed relatively evenly throughout the region of interest. The larger population, in this case, would be the thousands of potential sections from a block of tissue specimen. Stratified sampling procedures are often used to aid in solving this problem.¹⁰ In stratified sampling, the specimen is divided into a series of non-overlapping strata (for example, a 10-mm thick bone block might be divided into consecutive 1-mm thick bone layers) and specimens are then chosen from each stratum by simple random sampling.

C. CONTROLS

There are several different types of control groups, including normal controls, treatment controls and time controls. They are designed to act as comparisons for the experimental groups. A normal control is composed of a group of animals which does not receive the experimental treatment. Ideally, all characteristics, such as the strain, age and body weight, should be consistent between the normal control and treatment groups. They should also have the same living conditions and

feeding patterns. The objective is to vary only the factor or factors under investigation so that a valid comparison can be made between the two groups. Data obtained in the past or data from the literature can also serve as a normal control, but only if every factor involved is consistent with the current experiment.

1. Commonly Used Control Groups

For widely used species a considerable amount of normal control data can be obtained from the literature, such as the mechanical, histological, or biochemical characteristics of bone or cartilage. In bilateral experimental designs, the contralateral limb can be used as a treatment control but does not necessarily represent a normal control. This distinction is important. It is due to the fact that any abnormality created in the treated limb may potentially have indirect effects on the contralateral limb. An example of this is seen in a study by Frank et al.¹⁹ They demonstrated the abnormalities created in the contralateral knee of rabbits after unilateral transection of the medial collateral ligament. It has been hypothesized that the injury causes a shift of the weight-bearing burden to the contralateral limb. Another example is the systemic effect which is seen in the treatment control limb of carrageenan induced knee arthritis in the rabbit model (Table 1). The inflammatory arthritis not only causes reduced mechanical strength and elastic modulus of the long bones of the treated limb but also affects the contralateral limb (though less severely) and even the front limbs. The literature shows that treatment of one limb creates changes in the contralateral limb in a variety of different tissues, including: bone, cartilage, ligament, and muscle.¹⁹ The strength of a treatment control is that, by being a part of the same animal, it reduces the amount of unintended variation between the experimental and control groups. A contralateral (treatment) control is sufficient in many situations, such as: testing the effects of chemically induced arthritis or electrical stimulation on bone or cartilage tissue; comparing the efficacy of a new fracture fixation method to a standard method applied to the contralateral limb; comparing bone ingrowth into two different implant surfaces (one in each limb); and for examining the effects of immobilization on osseous and cartilaginous tissues.

Finally, many studies utilize a time control. In this type of design, each experimental subject serves as its own control. Measurements are made prior to administration of the experimental element and at consecutive intervals during the course of the study. Such would be the case in the study of a new drug which is intended to cause an increase in bone mineral density (BMD). Measurements of each individual's BMD might be made before the drug is given (baseline), and taken after 2, 6, and 12 months of treatment. At the conclusion of the trial comparisons can be made to the pretreatment levels. Alternatively, comparisons can be made between different subjects after various periods of treatment. For example, an examination of bone ingrowth into prosthetic implants may be undertaken. If a total of 30 experimental subjects are utilized, comparisons of the pushout strengths of the implants could be made after six, 12 and 24 weeks by sacrificing 10 of the subjects for each time period.

For some experiments, such as the first successful reconstruction of an amputated arm, controls are unnecessary or are impossible to design. There are also some situations which do not permit control of all variables. In these cases, the best available design should be applied with awareness of the design defects.

2. Unilateral and Bilateral Models

Bilateral animal models, also referred to as "paired designs," are used extensively (in 95% of the studies) for evaluating the biocompatibility and biofunctionality of foreign materials in bones.²⁰ In this type of design, one leg is used as the control side while the contralateral leg acts as the experimental side. One of the major advantages of using bilateral models is the more efficient (higher power for statistical analysis) comparison between control and experimental groups. The

variation for any given long bone in different animals is greater than the variation between paired bones in the same animal.² Therefore, a “paired left-right” comparison in a single animal is a closer comparison than between two different animals. “Paired” designs are based on the assumption that certain properties between bones of the left and right limbs are symmetrical. It has been previously established that the structure and mechanical properties of certain human and animal long bones are symmetrical. If conditions are well controlled, the number of animals in each group can be reduced significantly. When using an animal for which symmetry has not been experimentally established, a different approach is necessary. In this case the best design is to randomly designate the control and experimental side of the animal. By doing this, a potentially existing asymmetry between the two sides can be eliminated. The control and experimental side can then be compared using a paired Student t-test.

In some cases bilateral models are not appropriate. When major procedures are performed, especially those involving a joint, they create some degree of disability. If using a bilateral model, this may cause ethically unacceptable disability to the experimental animal.²¹ The situation is better tolerated if the animal has an untreated contralateral limb. This will allow the animal to heal over time and return to its previous level of function. In this type of situation, a unilateral model should be designed. Unlike human subjects, animals are not cooperative and unexpected complications occur. It is always wise to be a little conservative if potential risks have been foreseen. Common unilateral and bilateral animal models used in orthopaedic research are listed in Table 2, including published models and potential models.

3. Unicortical and Bicortical Plug Models

Special attention should be given to the use of transcortical plug models in dogs for studies of bone ingrowth into implants. Theoretically, a bicortical plug model can double the number of specimens for testing. By cutting the implant in the middle, one half can be used for histological examination and the other for mechanical testing. It is a good design if there are no major complications, such as fracture. However, the drill hole through two cortices reduces bone strength significantly, increasing the risk of fracture. This potential risk has been brought into serious consideration recently by our group. We recently had a catastrophic failure of experimental method resulting in two dogs being sacrificed at the beginning of a new project when femoral bicortical plug implantation led to bone fractures (Figure 1). The result was the loss of experimental animals without gaining significant scientific data. In addition, we had to deal with serious criticism and doubt from both the animal caretakers and the IACUC (Institutional Animal Care and Use Committee) although we have been qualified animal researchers for many years. A research group in Europe is known to have experienced a similar occurrence. On re-evaluation, the procedure may not be valid because it was only published by one research group and no details of the animals used (body weight, age) or animal casualties (any fractures due to the procedure?) were reported in any of the original articles.^{22–25} Therefore, a quick investigation has been performed on the effect of drill holes on the strength of the canine femur. The results show that a unicortical, 5-mm diameter drill hole in a canine femur creates a 38% reduction in bending strength, while a bicortical drill hole of the same size causes a reduction of over 50%. These results are basically in accordance with those of others (Table 3). A 50% reduction in bone strength is cause for concern, and thus the safety of the bicortical bone plug model should be carefully considered. The risk of fracture probably could have been minimized by using larger dogs, smaller diameter drill holes in areas of thick bone (i.e. close to the proximal or distal metaphyseal areas) and restrictive caging of the animals (limiting the cage space to prevent potential animal jumping). Carefully designed, a bicortical model has several advantages. It reduces the number of animals which are needed for an experiment and also has a greater statistical power. On the other hand, a bilateral unicortical femoral plug model as described by Bobyn et al.²⁸ appears to be safe although it does not have the statistical power of a

TABLE 2
Unilateral and Bilateral Animal Models Can Be Used in Orthopaedic Research*

Category	Model	Location	Animals Used
Unilateral model	Joint replacement	Hip	Rabbit, dog, sheep, goat, monkey
		Knee	Rabbit, sheep, goat
	Transarticular osteotomies	Knee	Rabbit, dog, sheep, goat, monkey
	Osteomyelitis	Tibia, femur	Rabbit, dog
	Prosthetic infection	Diaphyseal tibia	Rabbit
		Diaphyseal femur	Dog, rabbit
		Femoral condyles	Rabbit
	Segmental bone defect	Femur	Rat, rabbit, dog
		Tibia	Dog, sheep
		Radius	Rabbit, dog, rat
Arthritis (osteo-, inflam., infectious)	Bone lengthening	Tibia	Dog, sheep
	Knee	Dog, rabbit	
		Articular cartilage defect	Rabbit, dog
		Meniscal repair	Dog, rabbit, goat, sheep, monkey
		ACL reconstruction	Dog, rabbit, goat, sheep, monkey
		Knee collateral ligament repair	Rabbit, dog, goat, sheep, rat
		Tendon repair	Chicken, Rabbit, dog
		Nerve repair	Mouse, rat, rabbit, monkey (median nerve)
Bilateral model	Fracture healing	Femur	Rat, rabbit, dog
		Tibia	Rat, rabbit, dog
		Fibulae	Rat, rabbit, dog
		Radius	Rat, rabbit, dog
	Bone defect repair	Femur	Rat, dog
		Radius	Rabbit, dog, rat
		Ulna	Rabbit, dog
	Bone ingrowth to implant surfaces	Femoral condyles	Rabbit, dog, sheep, pig, rat, Guinea pig
		(plug, screw, intramedul. rod)	Upper tibia
			Rabbit, dog, goat, sheep, pig, rat
			Diaphyseal femur
			Dog,† sheep, rabbit, rat, pig, Guinea pig
			Diaphyseal tibia
			Dog, sheep, goat, rabbit, rat, pig
			Diaphyseal humerus
			Dog, rabbit, goat, sheep, pig
			Ulna
			Turkey

* Including published models and potential models.

† Trans-bicortical plug is not recommended.

bicortical model. Our intent is to notify researchers who may be considering the use of bicortical models that the risk of fracture is significant.

IV. EVALUATION METHODS

Chapter 6 summarizes some of the more useful evaluation methods in orthopaedic research, including clinical evaluation, necropsy, morphological or structural analysis, biochemical evaluation, mechanical testing, and the use of specialized devices or equipment. The most commonly used methods in orthopaedic animal research are: clinical observation, radiography, macro-observation at necropsy, histological evaluation, and mechanical testing. More sophisticated methods, such as electron microscopy, CT, and MRI, have many advantages but are expensive and often unnecessary.



FIGURE 1. Photograph showing a bilateral canine femoral fracture at the sites of bicortical plugs.

TABLE 3
Effect of Transcortical Drill Hole on the Mechanical Strength of Diaphyseal Bone

Subject	Bone	Bone diam. (mm)	Hole diam. (mm)	Hole diam./outer diam. of bone	Hole type	Mechanical test	Strength reduction (%)	1st author ^{Ref.}
Sheep	Femur	?	?	20%	Unicortical	Torsional	34%	Edgerton ²⁶
		19.6	?	50%	Unicortical	Torsional	60%	Hipp ²⁷
Dog	Femur	13.7	6	43.8%	Unicortical	4-pt. bending	37.6%	Authors' lab.
		13.7	6	43.8%	Bicortical	4-pt. bending	50.8%	Authors' lab.

A. SELECTION OF EVALUATION METHODS

The methods of evaluation should be carefully selected. Although many sophisticated and expensive testing methods have been described, the most useful techniques in animal studies are those which are simple, reliable, valid, efficient and economical. Most importantly, the test method must be valid, it must be capable of effectively measuring the parameter which it is intended to measure. The reliability of a method represents its ability to accurately and precisely measure the intended property. The accuracy of a measurement is the degree to which it represents the actual value of the measured parameter. Precision is a measure of the internal consistency of a measuring device, and represents the reproducibility of a measurement. A method can be extremely precise but inaccurate. For example, when evaluating a measuring caliper, if an object with a known thickness of 500 micrometers is measured and is consistently read as having a thickness of 600 micrometers, then the caliper is highly precise but is not accurate. An evaluation method must be both accurate and precise to be considered highly reliable.

The development of new evaluation methods requires evaluation of its accuracy. This involves comparison of the new test with a standard, which is usually a verified, well-established method. It is also very helpful to use at least two different techniques to demonstrate the accuracy of the results.

In order to test the precision of a method, repeated measurements of a known parameter can be made and then compared. Alternatively, the same procedure may be carried out by several different laboratory groups under standardized conditions, these results can then be compared.⁷ The results can then be analyzed for precision using Student's t-test or by correlation analysis.

B. COMMON SOURCES OF ERROR

Besides sampling error, inaccurate data can be the result of improper procedures during the preparation and conduction of an experiment (inadequate surgical procedures or specimen preparation), systemic error of testing systems, data collection error and personal error.

1. Procedural Errors

Procedural errors are those which result from inadequate experimental procedures, such as rough or inaccurate surgical procedures, insufficient or harsh decalcification of osseous tissues, or inappropriate specimen preparation for mechanical testing. For example, when cylindrical implants are to be implanted, the size of drill holes should be consistent. Large deviations in drill hole size will influence the amount of bone ingrowth. Inadequate preparation of tissues for histomorphological study will cause changed morphology or deviated quantitative parameters. Bone surfaces which are not perpendicular to the long axis of the cylindrical implant will result in altered mechanical values and inaccurate load-displacement curves.

2. Systemic Errors of Testing Systems

Systemic error is the inherent error of a measuring device, due to imperfections in its manufacture or modifications. This inherent error creates a relatively consistent difference between the obtained value and the true value. The obtained value is always on one side of the true value, either greater or smaller. All measurements are subject to some degree of error. For example, a significant machine compliance of a mechanical testing system will cause underestimation of the specimen stiffness.¹ Systemic error of a testing system can be corrected by careful calibration and zeroing. Also, it is important to realize that the inherent error of an evaluation method is almost always greater than that described by its inventors.

3. Data Collection Error

Missing data in a research project may pose many problems for investigators. In animal research, one common reason for loss of data is the reduced number of subjects caused by unexpected animal complications or deaths. Careless labeling of specimen or incorrect recording of data during an experiment are also potential sources of error.

4. Data Entry and Processing Error

Errors may also occur during the process of raw data entry into the computer, especially when many numbers or many categories are involved. Careful data verification procedures must be implemented during and after the input process. Data should be entered into the computer and verified by two different people. The use of computer software designed specifically for data entry may also reduce the chance for error by incorporating range checks, skip patterns and rekey verification into the data entry process.

5. Personal Error

Like the systemic error of a machine, some degree of personal error is inevitable. Most personal errors lie outside of the individual's awareness. Even the most conscientious, well-trained and ethical investigator is not completely free of error. Having an evaluation repeated by a second trained individual provides necessary verification and may also help detect the presence of bias. This is especially important when laboratory personnel have knowledge of the treatment of the animal and the expected results. Whenever possible, observations should be made by trained personnel blind to the treatment applications in an additional attempt to protect against bias and deliberate fraud.¹⁶

Reliable operator performance is the result of careful selection and training of personnel and time-sampling checks of the reliability of performance.⁷ It is the director's responsibility to test the reliability of each new technical assistant, preferably without the knowledge of the individual. The performance of any laboratory group has a greater chance of success when the team members place checks on each other and report all errors when they are detected or suspected.

V. DATA ANALYSIS

With the flourishing developments in computer technology, many software programs are available for statistical analysis.²⁹ State of the art computer software, highly tested for accuracy and efficiency, is now readily available for biomedical research and is cost effective. Statistica, StatView for Macintosh, and SAS (which is compatible with both Macintosh, Windows and mainframe systems) are some of the more efficient and widely accepted programs. Simpler programs such as StatWork and Excel are straight-forward and also adequate in many cases. Though these programs certainly

make statistical analysis less laborious, they are not foolproof. If the appropriate tests are not properly applied, the results will be misleading. Errors may also occur in the analysis phase due to incorrect calculations or data transformations.

The keys to successful statistical analysis are to have an effective experimental plan and an appropriate method for analysis. It is important to understand the strengths, weaknesses and underlying assumptions for each test. Inadequate or ineffective use of statistical methods is seen often, even in major medical journals, such as *J. Bone Joint Surg. (Br)*,³⁰ *Brit. Med. J.*,³¹ and *J. Bone Joint Surg. (Am)*.³² Due to the complexity of applying statistical testing techniques, it is strongly recommended that a statistician be consulted during the process of experimental planning, data analysis, and manuscript preparation.

Differences between experimental and control data may serve to support or refute an experimental hypothesis. Thus it is essential to determine whether the observed differences between groups are statistically significant or simply due to chance variation. There are a number of different statistical methods which can be used to determine statistical significance; each is appropriate in specific situations.

A. DESCRIPTIVE STATISTICS

A descriptive statistic is a number which is intended to summarize some characteristic of a larger source of data. Examples of commonly used descriptive statistical terms include: measures of central tendency (mean, median and mode) and measures of variability (minimum, maximum, range, standard deviation, standard error of the mean, and coefficient of variation).³³

The mean is the sum of the observations divided by the number of observations. Each observation plays a part in the calculation of the mean, so difficulties can arise if there are outliers in the data. Often, outliers are important parts of the data. Occasionally, however, the correction or disposal of outlying data can be justified, but only when obvious error is found in data collection or transfer. For example, an unusually weak pushout strength value may be omitted if it is found that the implant was improperly positioned during surgery (positioned mainly in medullary canal but not in cancellous bone bed as it is supposed to be).

The median is the central value in a set of ordered numbers. Unlike the mean, every number in a set does not enter into the computation of the median. The strength of the median is that it is not sensitive to the extreme scores in a set of data. It is appropriately used for analysis of data with a skewed distribution, such as the "time to recurrence." The mode is simply the most frequently seen value in a set of data. It is most useful for demonstrating the clustering of values in a set of data.¹² The minimum value is the smallest value in a set of data and the maximum value is the largest one. The range is the absolute value of the difference between the minimum and the maximum values. These are simplified expressions of the variability of a set of numbers. Often they do not accurately represent the rest of the dataset, but they are easy to document and interpret.

A more precise calculation of variability is the standard deviation (SD). It represents the average deviation from the mean of the individual observations. SD is valid when the data falls in a normal or Gaussian distribution. The standard error of the mean (SEM) is a statistic that estimates the central tendency of the data or the variability of the sample mean in the population (when repeated samples of the same size are taken from the population). It is calculated by dividing SD of the observations by the square root of the number of observations. SD is often used when the purpose of an observation is to compare two means such as the means of mechanical strength of bone in control and treated groups. SEM is more useful when the goal is to determine the range of a normal value in a population such as white blood cell count in a human population or the mechanical strength of human bone.

The coefficient of variation (CV) is a unitless expression of variability. It is calculated by dividing the sample standard deviation by the sample mean. It is especially useful when comparing the variability of several different measurements, or when measurements are made in different

units. Normally, if a CV value is under 0.2, it indicates a relatively low variability. Values less than 0.1 indicate a very small deviation or they may indicate a sufficiently large sample size. The limitation of CV arises when the mean is very small (close to 0). In this situation the CV value will be quite large (since the mean is in the denominator of the equation) and may not accurately represent the variation of the dataset.

B. ONE SAMPLE ANALYSIS

One sample analysis is used when it is necessary to compare the average of some parameter in an experimental group to a generally accepted or hypothesized parameter (rather than comparing it to a control group). The most commonly used method for one sample analysis is the one sample t-test. It compares an experimentally determined sample mean to a hypothesized mean for the population, and determines the likelihood that the observed differences between the two means occurred by chance alone. The results of such a t-test are reported in the form of a p-value, which ranges from 0 to 1. A p-value approaching one indicates a high likelihood that any difference between the sample mean and the hypothesized mean is purely due to chance and is not considered significant. On the other hand, a low p-value, such as 0.05 (a commonly used limit) or smaller, indicates that the difference between the two means is significant and is probably not due to chance alone.

C. UNPAIRED COMPARISONS

The unpaired student t-test is used to assess the difference between the average measurements of two separate groups. This might include comparisons between an experimental and a control group or between two different experimental groups. For example, one might want to determine whether a new drug causes an increase in bone mineral density. An experiment might be arranged such that an experimental group is treated with the new drug, while the control group receives a placebo. After a sufficient length of time, the BMD of each group is measured. The student t-test would be applied to statistically test for a difference in the means of the two groups. The results are expressed in p-values, as previously described.

D. PAIRED COMPARISONS

Paired comparisons serve to compare two sets of data which are naturally paired in some way. The most common scenario is a comparison of two separate sets of data taken from the same experimental group at different times or under different conditions. An example of this type of experiment would be the evaluation of the effectiveness of a muscle stimulator in improving strength. Measurements of strength might be taken before application of the muscle stimulator (baseline), after six weeks of use and after 12 weeks of use. Another typical situation is a study which utilizes a bilateral experimental model to evaluate the ingrowth of osteopenic bone into a prosthetic implant, and compares it to the “normal” bone ingrowth in the contralateral femur. Paired comparisons are generally more powerful than unpaired ones, since each experimental subject serves as its own control. The results can be analyzed using the paired t-test or, for two or more repeated measurements, the repeated measures ANOVA. The results are again reported as p-values.

E. ANALYSIS OF VARIANCE (ANOVA TEST)

ANOVA is a statistical tool which may be used to evaluate the impact of one or more nominal independent variables on a continuous dependent variable. A detailed discussion of the theoretical basis of ANOVA is beyond the scope of this book. In simplistic terms, ANOVA makes comparisons of the variations within and between experimental groups, and by doing this it determines whether group means differ from each other (Figure 2). Groups with similar means tend to produce a

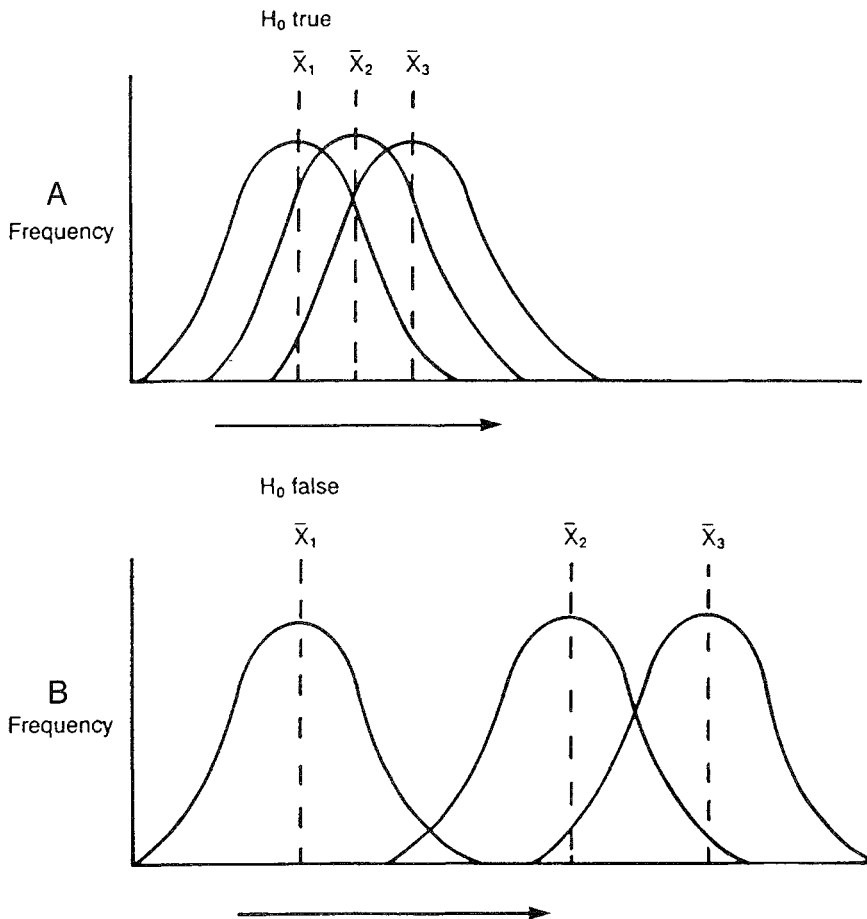


FIGURE 2. Figure A illustrates the relatively small amount of intergroup variance which is present when group means are not significantly different. In Figure B the group means are significantly different, consequently there is a greater intergroup variance. (From Reference 12, with permission)

relatively small intergroup variance (variance among all groups), whereas groups with significantly different means create a relatively large intergroup variance. One strength of ANOVA is its ability to simultaneously compare multiple independent variables. For example, a study might be undertaken to determine whether or not the region of the country in which someone lives (nominal independent variable) has an effect on their risk of osteoporosis (dependent variable). ANOVA allows one to compare the rates of osteoporosis of individuals in the Northeast, South, Midwest and West Coast regions to determine if there are differences based on location. If differences are observed using ANOVA, post-hoc methods such as Fisher's PLSD, Scheffé's F and Bonferroni/Dunn procedures can then be applied to examine differences between various pairs of data.

F. CORRELATION AND REGRESSION ANALYSIS

Correlation analysis is a tool used to indicate the degree of linear relationship between two variables. This relationship is expressed as a correlation coefficient, with a value ranging from -1 to $+1$. A correlation coefficient of 0 means that no linear relationship exists between the two variables. A value of $+1$ indicates a strong positive correlation. This reflects a linear relationship between variables such that when one variable increases, the other increases to a proportional

degree, and when one decreases, the other decreases to a proportional degree. A correlation coefficient of -1 , while still indicating a linear relationship, connotes a strong negative correlation. When one variable increases the other decreases to a proportional degree, and vice versa. Correlation coefficients might be used to describe the relationship between bone density and mechanical strength.³ As BMD increases, the mechanical strength of the bone increases to a proportional degree. This indicates a strong positive correlation between the two. Correlation analysis measures only the linear relationship between two variables and it should not be used if the relationship is nonlinear. A scattergram may be used to determine whether or not a relationship is linear.

Regression analysis is used to predict the value of a dependent variable based on the value of one or more independent variables. It can be applied only when: (1) there is a linear relationship between the independent and dependent variable, (2) all variables are continuous, and (3) all values are independent of each other. In reference to the previous example, regression analysis might be used to predict the mechanical strength of a bone (dependent variable) based on its BMD (independent variable).

G. NONPARAMETRIC DATA

Nonparametric tests are statistical techniques which can be applied when there is no assumption of a Gaussian distribution within the population. Thus they are often referred to as distribution-free tests. While not as powerful or flexible as their parametric counterparts (i.e. t-test and ANOVA), nonparametric tests can be applied in situations where parametric tests are not valid. There are a number of nonparametric tests. Chi-square is the most commonly used and it requires nominal data. Others, which require ordinal data, include the Mann-Whitney U test, the Wilcoxon Signed Rank test, and the Kruskal Wallis test. The Mann-Whitney U test is used to test the hypothesis that the distributions of two different sets of data are equal. It is the nonparametric equivalent of an unpaired t-test. Similarly, the Wilcoxon Signed Rank test is the nonparametric equivalent of the paired t-test. The Kruskal Wallis test is analogous to ANOVA and tests whether two or more sets of data come from the same distribution or from different distributions. Further discussion of their uses may be found in many statistical texts such as those by Abacus Concepts, Inc.,³³ Munro et al.,¹² and Forthofer and Lee.¹⁸

VI. PUBLICATION

The functions of a scientific paper are to present new research methodologies, the results of a scientific project, or new theories derived from the results. How well these functions are carried out depends on their presentation in the manuscript. Detailed information on how to write a scientific article can be found in books by Leedy,⁸ Hawkins and Sorgi,⁹ Bay,³⁴ O'Connor,³⁵ Garb,³⁶ and Whimster,³⁷ or in papers by Morris³⁰ and the International Committee of Medical Journal Editors.³⁸ The following are some brief guidelines for writing a successful paper.

A. TITLE PAGE

The title page should include the following information: (1) the title, which should be both concise and informative; (2) full names and academic degrees of all authors; (3) the affiliation and location where the project was conducted; (4) information about the corresponding author, including address and contact numbers; and (5) a short running title (no more than 40 characters).

B. ABSTRACT

An abstract is a condensed form of the paper. Included in it are the research hypothesis, the experimental purpose and plan, the methods, the results, and conclusions. Most importantly, an abstract should be self-contained and should give readers a concise overview of the entire paper.

To ensure a self-contained quality, no unspelled abbreviations should be used. The appropriate number of words for an abstract is 250–350. If the abstract is oversimplified it is virtually useless, while an excessively lengthy abstract makes it difficult for the reader to grasp the main points of the article. According to the requirements of publishers, 3–6 key words should be supplied at the end of the abstract to assist indexers in cross-referencing the article. This indexing information may also be published with the article.

C. INTRODUCTION

A clear statement of the question to be answered, the hypothesis, and the experimental purpose should be given in the introductory section. Often, a brief review of the history and a discussion of any recent developments in the field are given so that readers can easily understand the questions. For projects without a clear hypothesis, a clearly stated question and purpose are essential.

D. MATERIALS AND METHODS

The materials used and experimental protocol should be discussed in detail. Information concerning the animal species used should be given, including strain, sex, age, body weight, number of animals (or specimens), housing conditions and state of health.³⁹ Failure to include this information makes comparisons between studies very difficult or impossible. Examples include comparisons of mechanical strength of bone or cartilage.

Any new materials, drugs, devices or methods used in the study should be presented in detail. The sources, including the names and locations of the companies, of any major supplies or materials used in the experiment should be indicated. For previously described methods, properly published references should be cited. Meeting abstracts, local proceeding papers, or papers from a rare book are inappropriate sources to reference since they are not widely available. After a reference for a method is cited, a brief description of the method should be given in order to fully relate the content of the study. A detailed description of the surgical approach, technique, surgical procedure and performance of it must be included in surgical studies. Also, the sampling procedure, sample size determination, the measures used for reducing errors, and the statistical methods used for data analysis should be included in this section of the manuscript.

E. RESULTS

In the results section, a detailed description of the findings should be given in the form of text, tables, graphs, line drawings or photographs. A combination of the above-mentioned components can be used depending on the nature of the data. The precision of the measurement techniques and observed results (confidence intervals, standard deviation or standard error) should accompany the presentation.

For newly developed animal models, any casualties or complications associated with the procedure should be indicated. It is uncommon for a new model to be developed without any casualties or complications. These casualty reports are frequently left out of the papers. In a recent review of the methods sections of biomedical research papers, 30% failed to mention the total number of animals used, and animal deaths were not always recorded.³⁹

Some reviewers have suggested that for a single set of data either a table or a graph should be used, but not both. The authors do not necessarily support this view. If the values in the data set are relative or standardized numbers, only a graph is capable of effectively showing the difference or trend. On the other hand, although a graph is good for catching the reader's eye, a table containing the actual values is more informative. A table of actual values allows easier comparison among studies from different groups. When only relative values are included in a study, as in the correlation between mechanical properties and structural or biochemical parameters, comparisons between studies are difficult if not impossible.

F. DISCUSSION

The discussion section should include an explanation of the results obtained from the current study and the conclusions or proposed theories which are drawn from the results. Results may be discussed and compared with other particularly relevant studies and topics. Opinions on the validity of the data and the reliability of the testing methods should be given here. Existing data in the literature should be used for comparison and support for the new data or theory. If applicable, this section may also mention the of clinical relevance of the results. The discussion is normally completed with a summary of the major conclusions. In some journals the conclusions are given in a separate section. Most authors feel that conclusions should not be stated, especially in the abstract, unless they are completely justified by the results.³⁰ Lengthy discussion is usually unnecessary and makes it difficult for readers to focus on the salient points of the article. Most readers prefer a simple, straightforward, and relevant discussion section.

G. REFERENCES

The references should be relevant, current, and complete. Excessive use of references is not appropriate for an original paper. More references should be used for review papers but they also should be representative, relevant, and current. Outdated references are difficult to find and frequently are useless. Older references should only be used to bring in original theories or methods or for supporting the theory that the authors are going to propose. Included in the reference should be: the authors (up to three or five), editors, title, journal name, book title, volume, edition, page number, publisher, location of the publisher and year of publication. Every reference needs to be verified with the original paper or by a MedLine search before the paper is submitted for publication.

VII. RESEARCH ETHICS

A. ETHICAL USE OF LABORATORY ANIMALS

Due to public concern for animals' rights and welfare, Russell and Burch, two English scientists, introduced *The Principles of Humane Experimental Technique* in 1959.⁴⁰ They described the principle of the "three Rs": (1) replacement of animals in biomedical research, by using *in vitro* methods or by using animals that are phylogenetically more primitive; (2) reduction of the number of animals required; and (3) refinement of experimental methods to reduce the ethical costs in terms of painful or stressful procedures. Today, in North America, most European countries, and several other developed countries, specific regulations and guidelines have been established for the proper use and care of laboratory animals. Researchers should comply with all regulations and guidelines available in their own countries and should promote the development and perfection of them. See Chapters 1, 2, and 5 for more information on the ethical use and care of laboratory animals.

B. OBLIGATIONS OF RESEARCHERS

In a 1992 paper, Manly summarized the four obligations of researchers: obligations to the society, obligations to sponsors or employers, obligations to colleagues, and obligations to human subjects.¹⁰ Although the obligations to society and human subjects are not directly applied to animal research, achievements in animal research will eventually serve human patients. Therefore, researchers are expected to deliver the highest quality of work to the public. The investigator should follow a particular code of ethics for the design, analysis and reporting of a study. In certain cases, project information should be kept confidential and not exposed to other researchers without permission from the principal investigator or sponsor. The sponsor should be informed of any significant changes in an ongoing project and any major problems should be reported, as well. The investigators should have a realistic idea of their level of ability and the probability that a project can be carried

out successfully. Most importantly, investigators should be truthful about their research results and shortcomings so that other researchers can learn from them and ultimately promote continued development in the field. Fabricated experimental results and incorrect information are detrimental and may mislead others' studies, resulting in the waste of research funds and scientific impurity.

C. AUTHORSHIP AND ACKNOWLEDGMENT

The senior investigator is responsible for ensuring that the intellectual contribution (conception, design, improvement of methodology, analysis and interpretation of data, and the final approval of the paper) and the amount of effort (conducting experiment, collecting data, and drafting illustrations or methodology section) by co-authors are reflected in the authors' designation or sequence in the authors' list. It is very important to ensure that all authors desire to be included as such. Corresponding or senior authors are those who contribute original ideas, execute the experimental design, and draw conclusions or theories from the results. General supervision of the research group or participation solely in the acquisition of funds does not justify authorship. Corresponding or senior authors are also responsible for the correct spelling of co-authors' names. All authors should also be willing to assume public responsibility for its content, although for some journals only corresponding or senior authors are held accountable.

Acknowledgments of appreciation to those who helped on the project should be included. The names of the financial supporters should also be clearly stated to demonstrate the gratitude of the authors and to prevent any potential conflicts of interest.

D. INTELLECTUAL PROPERTY

The term "intellectual property" was formally defined in the 1967 Stockholm Convention, which established the World Intellectual Property Organization. Article 2 (viii) of the convention provides that "intellectual property" shall include the rights relating to: literary, artistic and scientific works; performances of performing artists, phonograms and broadcasts; inventions in all fields of human endeavor; scientific discoveries; industrial designs; trademarks, service marks and commercial names and designations; protection against unfair competition; and all other rights resulting from intellectual activity in the industrial, scientific, literary or artistic fields. Protection of intellectual property is the subject of many international agreements.

The development of new techniques in the field of biotechnology has led to the emanation of such treaties as the *Treaty on Intellectual Property in Respect of Integrated Circuits*, signed in Washington in 1989 and the *Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure*, signed in Budapest in 1977. Increases in the trade of intellectual property over the past decade has made it apparent that there is a compelling need for further international cooperation in this area.

Protection of international property rights is now the subject of much interest and debate on the local and world stage. International agreements concerning intellectual property protection afford inventors only limited patent protection in certain circumstances. It has become apparent that more comprehensive and formal agreements are needed in the area of biomedical research. When an article is published in the literature, the copyright is generally transferred from the author(s) to the publisher for a certain period of time.

Authors should respect the originality of others' theories and methods by referencing their original papers or by explicitly indicating the sources (such as personal communications or ideas from journal manuscripts or grant proposals reviewed by the author). It is unethical for investigators to take original ideas from others' manuscripts or grant proposals and present them as their own. Original theories or methods which impact science must be distinguished from common knowledge, and the former must always be cited.

E. INTEGRITY

All data with informative detail should be clearly presented to the readers. Although outright fraud is a rare occurrence, simply avoiding mention of important facts may also lead to disastrous consequences when others attempt to repeat an experiment or use the described procedures. A typical example is the lack of data on animal casualties during the process of developing a new model.³⁹ Even an occasional complication may indicate significant risks for a proposed model. The weaknesses of a project should be addressed openly. It is also important to discuss any factors which may have adversely affected the results. Mention of them should allow the problems to be avoided in the future.

F. NEVER DUPLICATE PUBLICATION

It is unethical to submit the same article to more than one peer-reviewed journal simultaneously and it is even worse to have the same paper published in two different journals. This type of unethical practice in publication can be easily recognized with the computerized literature searches (such as MedLine) which are now available. It is acceptable to submit the paper to a second journal only after it has been rejected by the first one. It is also unethical to publish a study based on a certain set of data, and then to publish a followup article without adding a significant amount of new data or information. Finally, one should not publish two different versions of the same paper in order to publish in more than one place.

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3 Animal Selections in Orthopaedic Research

Yuehuei H. An and Richard J. Friedman

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I. WHY USE ANIMAL MODELS IN ORTHOPAEDIC RESEARCH?

Animal models provide important knowledge of pathological conditions that can eventually lead to the development of more effective clinical treatment of diseases in both humans and animals. Research using animal models acts as the bridge between *in vitro* studies (such as studies of protein adsorption, cell adhesion and toxicological tests) and human clinical trials. It is an essential research tool which is applicable for many biomedical projects.

When making a decision on whether or not an animal model should be used for a particular study, the following questions should be raised: (1) Can the knowledge gained from the animal model be extrapolated to human conditions? (2) Are there any alternative methods which can verify

the hypothesis? (3) Does the procedure cause extraordinary pain or disability to the animals? An *in vivo* study using an animal model may be considered necessary and appropriate if the procedure does not cause extraordinary pain or disability to the animals, the knowledge gained can be applied for the benefit of humans or animals, and there are no other *in vitro* alternatives.

For example, in the development of a new bioabsorbable material, measurements of its mechanical strength and degradation rate in a saline environment must be tested. Its biocompatibility in cell culture must also be assessed (Figure 1). After these *in vitro* studies, the material cannot immediately be tested in humans because it may have undetected toxic effects in human tissues. In the past, some products containing polylactide acid (PLA) and polyglycolic acid (PGA) have caused tissue lysis in human subjects leading to aseptic abscesses. Will the new absorbable material cause a similar problem? This question has to be answered before clinical trials are undertaken. Therefore, an animal model (using lower level animals such as rats) can be used to test the biocompatibility and degradation rate of the material, *in vivo*. Tests such as these are normally accomplished by subcutaneous and intraosseous implantation. If the experiment does not reveal any significant toxic effects, a second animal model (using higher level animals such as rabbits) can be used to evaluate the potential applications of the material, such as fixation of fractures or osteotomies or repair of ligaments or cartilage defects. At the same time, the process of material degradation and replacement by host tissues could be investigated. If the material functions well enough for fracture fixation or ligament repair in all animal models, then consideration may be given to a cautious, well controlled human trial (Figure 1).¹

II. HOW TO CHOOSE ANIMAL MODELS IN ORTHOPAEDIC RESEARCH

A. ETHICAL AND GENERAL CONSIDERATIONS

1. Ethics

Scientists today must realize that the use of animals in biomedical research has become an ethical issue. Although a small number of people feel that animal experiments should be stopped altogether, most have more moderate views. They recognize the need for animal research but feel that certain limitations and regulations are necessary. In general, the use of phylogenetically primitive animal species is more readily accepted by the public, so research with invertebrates is preferred over the use of vertebrates. Similarly, the use of pigs, rabbits and goats is preferred over the use of animals such as dogs or cats, to which humans are emotionally attached. Those studies which are directly applicable to human patients are also more well-accepted than studies with primarily basic scientific value. The amount of discomfort experienced by the animals is another important element. While it may be possible to justify experiments which cause slight harm or distress to the animals, those which create severe pain and suffering are usually not acceptable. A limited number of animals should also be used. Because of these factors, the choice of animal species in any given experiment is often the result of compromise. Ideally, scientific judgment should be the deciding factor; however, public concerns are also important and sometimes outweigh the scientific merit of a study. It is the authors' opinion that rats, rabbits, goats and sheep should be considered first in a new project. For most orthopaedic animal studies, there is no specific reason for dogs to be used when goats and sheep are also available.

2. Availability

Sometimes, the process of selecting animal species for a study could be further influenced by the availability of animals, boarding requirements, and cost. Generally speaking, securing animals of a particular species, strain, type, or age is very important in the selection of experiment animals. In some developed countries such as the United States, the availability of commonly used animals in orthopaedic research is not a problem. You can obtain rats, rabbits, dogs, goats or sheep any time during the year. You may want to contact the animal resources early enough if an animal

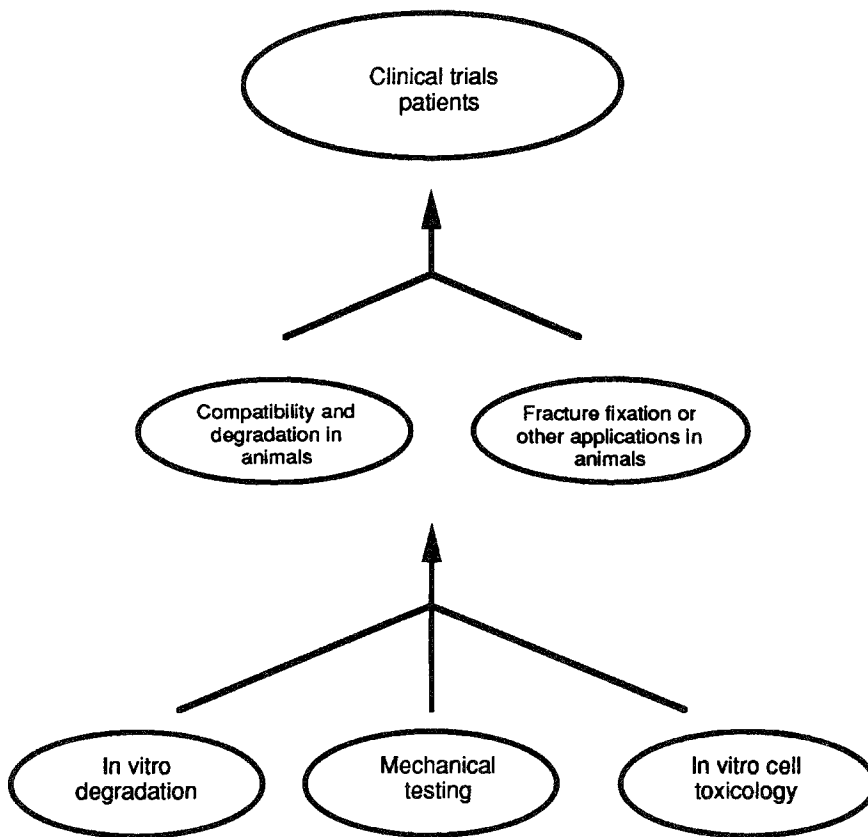


FIGURE 1. Schematic drawing of the steps in the development of a bioabsorbable material for clinical application.

species of a particular age or a rare species such as a primate is required. In many developing countries, availability is still a major problem because of the lack of standardized laboratory animal resources.

3. Housing Requirements

At certain institutions, the animal research facility may not have the capacity to house certain animal species. Then, the researcher may want to select another animal species if he or she does not want to travel to other facilities. More often than not, the latter is not really a better idea than choosing another species instead if it is acceptable for the project. Often, the researchers do not have a chance to inspect and treat the animals, leading to less reliable data. Also, doing a project at a commercial facility or one with a long traveling distance is very costly.

4. Ease of Handling

One reason more rats or rabbits than goats or sheep are selected in biomedical research is the easy handling of these animals. Large animals are good for certain investigations requiring large sized bones or other tissues, such as ACL reconstruction using ligament prosthesis designed for the human knee. Large animals create more difficulties than small animals for transportation, housing, peri-operative care, specimen handling, and disposal.

5. Cost

The costs of animal purchasing, transportation, quarantine time, housing, unexpected loss, surgical supply or services, or special equipment such as CT or MRI should not be decisive factors in selecting experimental animals; however, the costs of these items should be carefully calculated before embarking on the project (Table 1). Also, financial limitations such as the size of the grant or the definite amount of money available for the project are important factors in selection of animals. The bottom line is that you can use a cheaper animal as long as you believe the data derived from that animal species are valid to verify the hypothesis.

6. Susceptibility to Disease

Spontaneous diseases in animals during experiment can seriously compromise the experimental plan, confuse research data, and raise the cost (Table 1). Researchers should avoid using animal species known to have high incidence of a particular disease. For example, conventional rats are susceptible to chronic respiratory disease and conventional rabbits are likely to have *Pasteurella multocida* infections. Because of their low costs they are often used for acute procedures. If the study is chronic and long survival (more than a month), SPF (virus and antibody free) animals should be selected.

B. AVAILABLE BACKGROUND DATA OF THE ANIMAL

A brief survey of the animal models used in the research papers published in *J. Orthop. Res.* in 1992 to 1996 was conducted to show the preference of researchers on the use of animal subjects in orthopaedic research (Figure 2, Table 2). The results showed that the most commonly selected animals are rabbits, rats, dogs, and goats. One should be aware of the limitations of this survey (one particular time period for only one journal).

First, the existing research data in the literature for an animal species, such as anatomy, physiological features, or the responses to drugs and surgical procedures, especially the information relevant to orthopaedic research, are very important for the selection of research animals. From Table 1, it is very clear there are much more background data for rabbits, dogs, rats, and goats than for the other animals, which reflect the value and suitability of these animals. Second, the existing data help researchers to repeat or extend previous work because they need the information of the species, strain, and basic biological data of the animal and the corresponding surgical procedures employed. Furthermore, the basic data help veterinary doctors and animal caretakers to better maintain the animal during the research period. These considerations have been influential in the fact of wide usage of only the few species shown in Figure 2. However, it is encouraging now that goats and mice are becoming valuable research subjects in the field. From an ethical point of view, it is easier for people to accept animals other than dogs for use in research.

C. COMMONLY USED ANIMALS IN ORTHOPAEDIC RESEARCH

1. Rabbits

The rabbit is one of the most commonly used animals in orthopaedic research. The above-mentioned *J. Orthop. Res.* survey showed that rabbits were used in 26% of the total animal studies (45 out of 171). Also based on the relevant publications (through the MedLine search), rabbits are more suitable for the studies of articular cartilage repair, ACL or medial collateral ligament (MCL) reconstruction,² fracture or osteotomy, bone ingrowth,³ bone defect repair, steroid-induced osteonecrosis,^{4,5} or osteoarthritis.⁶ With the increasing interest on articular cartilage repair using tissue engineering techniques, the rabbit femoral articular defect model has become more and more popular in the last five years.⁷⁻⁹ Although not shown clearly in the JOR survey (Table 2), rabbit

TABLE 1
Common Animal Species Used in Orthopaedic Research

Animal	Lifespan (Years)	Common disease	Average weight (kg)	Cost/purchase (\$*/per animal)	Cost/housing (\$/day/animal)	Housing requirements	Ease of handling
Rat (conventional)	2-3	Multiple infections	0.2-0.4	15-25/each	0.20	Nothing specific	Easy
Rat (VAF† or SPF‡)	2-3	None	0.2-0.4	15-25/each	0.20	Temperature/moisture controlled room	Easy
Rabbit (conventional)	7-8	Pasteurellosis	3-5	25-35/each	1-2	Temperature/moisture	OK, one person
Rabbit (SPF)	7-8	None	3-5	35-55/each	1-2	Temperature/moisture controlled room	OK, one person
Dog	10-12	Heart worm Canine hepatitis	10-25	300/each	4-5	Large cage	OK, two persons
Goat	10-15	Bacterial or viral pneumonia	50-70	250/each	4-5	Nothing specific	Difficult, two persons
Sheep	10-15	Bacterial or viral pneumonia	50-70	250/each	4-5	Nothing specific	Difficult, two persons

* \$: US dollar; † VAF: Virus antigen free; ‡SPF: Specific pathogen free.

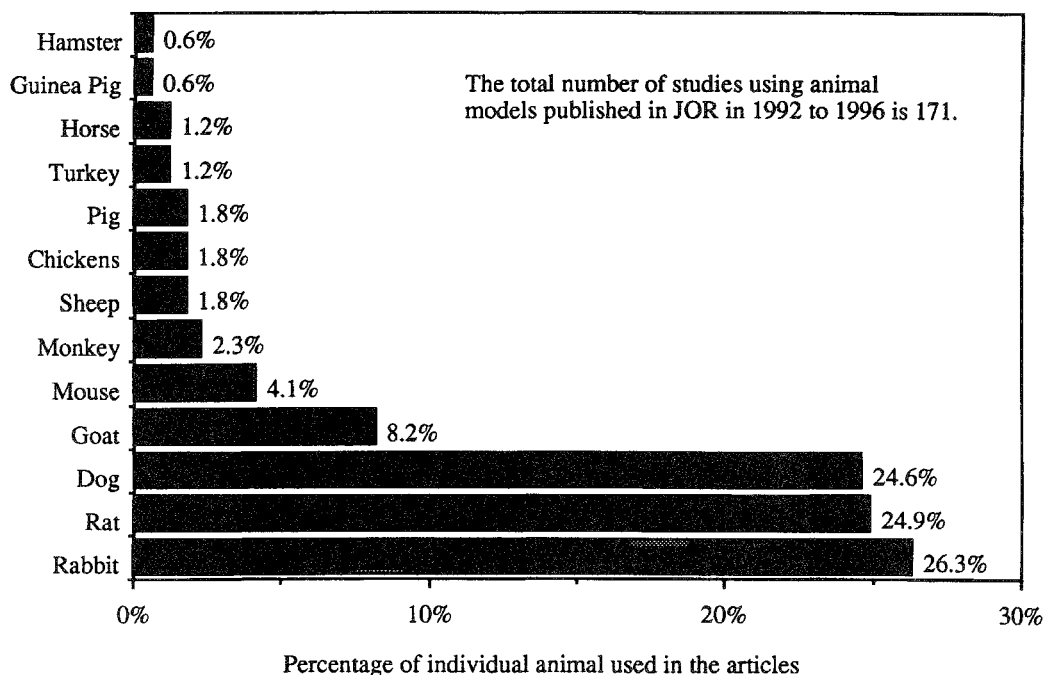


FIGURE 2. The animal subjects used in the research papers published in *J. Orthop. Res.* in 1992 to 1996.

bone defect models, such as radial^{10,11} or cranial defects,^{12,13} have been well established. Many researchers used rabbits for the studies of inflammatory arthritis and local osteopenia.¹⁴⁻¹⁶ Rabbits are also successful subjects for the studies of osteomyelitis,¹⁷ septic arthritis,¹⁸ and foreign body or prosthetic infection¹⁹ since they seem to be more susceptible to bacterial infection than other animals such as rats. In the authors' laboratory, the rabbit distal femur plug model has been used successfully for the studies of bone ingrowth to implants with different surface coatings or textures.^{16,20,21}

Above all, the rabbit is a relatively high level vertebrate, having a good size which enables easy surgical operations and convenient radiographic, histologic and mechanical analysis and it is relatively economical compared to dogs. One shortcoming of using rabbits is they seem to be more fragile than rats and dogs. There are more unexpected deaths caused by complications or diseases in rabbits.

2. Rats

The rat, a rodent, has a mean healthy lifespan of 21-24 months. Bone elongation ceases by age 6-9 months, an age after which considerable useful experimental lifespan remains.²² Although the rat is a lower level vertebrate compared to the rabbit, dog and goat, it is another most popular animal subject in orthopaedic research for its low cost and easy handling. It plays a very important role in the field, such as the studies of fracture,²³⁻²⁵ bone defect repair,²⁶⁻²⁸ bone ingrowth,^{29,30} bone or joint infections,^{31,32} osteoporosis,^{22,33,34} osteomyelitis,³⁵ bone circulation,³⁶ prosthetic debris,^{37,38} biocompatibility (subcutaneous or intramuscular implantation of biomaterials),^{39,40} hemocompatibility of vascular prosthesis,⁴¹ or nerve repair.^{42,43} It seems to be a universal animal subject since rats have been used everywhere in orthopaedic research. Due to all of its advantages it is not unreasonable to consider the rat first as the animal subject for a new project.

TABLE 2

The Animal Models Used in the Research Papers Published in the *Journal of Orthopaedic Research* from 1992 to 1996 (Total: 171 Articles)

Tissues	Models	Rabbits	Dogs	Rats	Goats	Mice	Monkeys	Sheep	Chickens
Bone	Fracture/osteotomy	4	1	5	1			2	
	Bone defect/bone substitute	3	8	4					
	Subcutaneous bone substitute			5					
	Bone ingrowth	2	7	2	1		1		
	Bone biology/growth	1	2	3					
	Infections of bone, joint, or implant	3		3					
	Prosthetic debris			2					
	Limb lengthening/bone distraction	3			1				
	Electrical property/electrical stimulation	1	1						
	Bone tumor			2		2			
Joint	Osteoarthritis		4				2	1	
	Inflammatory arthritis	2	1	2		2			
Cartilage	Cartilage biology or repair	7	4	1	1	1			
Ligament & tendon	ACL reconstruction	6			9		1		
	MCL reconstruction	7		2					
	Tendon repair	2	3	3					3
	Soft tissue subcutaneous biocompatibility			1					
	Blood vessel/blood flow	2	5		1				
	Nerve repair	1		2		1			
	Muscle	1	1	2					
Others				5		1			
Total		45	42	41	14	7	4	3	3

3. Dogs

The dog is a higher level vertebrate. It has probably the closest *in vivo* condition to the human except for nonhuman primates. Dogs have played a dominant role in orthopaedic research. This animal has been used extensively and successfully in the studies of fracture,^{44,45} bone defect repair,⁴⁶ bone ingrowth,^{47,48} prosthetic infections,^{49,50} osteomyelitis,⁵¹ prosthetic debris,⁵² osteonecrosis,⁵³ osteoporosis,^{22,33} osteoarthritis,⁶ ACL reconstruction,² meniscal repair and reconstruction,^{54,55} cartilage biology or repair, spinal procedures, or bone vasculature and blood flow.

4. Goats

Goats are becoming popular and valid animal subjects in orthopaedic research in recent years, especially for their role in the research of ACL^{2,56} and bone ingrowth.^{57,58} They also have been used for the studies of biocompatibility,⁵⁹ joint replacement,⁶⁰ fracture,⁶¹ limb lengthening,⁶² meniscal repair,^{63,64} or cartilage biology or repair.^{65,66} It is a newcomer compared to dogs and lacks basic data. Due to their nature as a higher level vertebrate and non-pet status compared to dogs, goats will play a significant role in orthopaedic research in the future. The shortcomings of using goats include difficulty of handling and requirement for large housing space.

5. Sheep

The sheep is a large animal similar to a goat, which has both growing and adult skeletal phases, but the age of peak bone mass is not clear. There is less information about sheep as a subject for

orthopaedic research compared to dogs and goats, but the available data are increasing in recent years. It is common to notice sheep as the selected animal in the literature such as bone defect repair,^{67,68} osteoporosis (questionable),⁶⁹ meniscal repair,⁷⁰ cartilage defect repair,⁷¹ osteoarthritis,⁷² ACL reconstruction,^{73,74} vessel graft,⁷⁵ nerve repair⁷⁶ or limb lengthening.⁷⁷

6. Mice

The mouse is a small rodent, which has become more and more popular in skeletal research because of the ease with which its genome can be manipulated and investigated. Both regular mice or nude mice have been widely used in the studies of osteogenesis in bone or soft tissues,^{78–80} chondrogenesis of potential materials in subcutaneous tissue,^{81,82} osteoporosis, inflammatory arthritis, bone tumor, or nerve repair. The most common use of the mouse is the screening of potential substances for osteogenesis or chondrogenesis.

7. Primates

Nonhuman primates have both growing and adult skeletal phases, and the peak bone mass occurs at age 10–11 years. From a scientific point of view, primates are ideal for biomedical research because among animals they are the closest to humans. They have been used in the studies of osteoporosis,^{22,33,34} bone ingrowth,⁸³ bone repair,⁸⁴ cartilage repair,⁸⁵ or osteoarthritis.⁸⁶ Because of the lack of availability and high cost, the use of primates has been limited to the research projects for which they are definitely necessary, such as the evaluation of new potential drugs for osteoporosis.^{22,33,34}

8. Pigs

The pig has both growing and adult skeletal phases. It has been reported as a subject for the studies of effects of exercise on the skeleton,⁸⁷ osteoporosis (more work needed for its validity),²² post-traumatic osteonecrosis of the femoral head,⁸⁸ fractures of cartilage and bone,⁸⁹ and bone ingrowth in the metaphyseal plug model.⁹⁰

9. Horses

The horse is the largest animal used as an experimental subject. It has been used mainly for studies of cartilage or joint conditions since there is rich cartilage tissue in the horse. It has been reported as an experimental animal for articular defect repair,^{91,92} experimental synovitis,⁹³ and septic arthritis.⁹⁴

10. Other Animals

Guinea pigs are very popular for studies of osteoarthritis.^{95,96} They are also used for a model of post-traumatic osteomyelitis.⁹⁷ Other animals are sometimes used, such as cats for osteoarticular transplantation,⁹⁸ hamsters for implant infection,⁹⁹ chickens for studies of scoliosis¹⁰⁰ or tendon repair,¹⁰¹ or turkeys for bone remodeling.¹⁰²

D. COMMONLY USED ANIMAL MODELS IN ORTHOPAEDIC RESEARCH

According to the literature and the authors' experience the suggested animal models for common orthopaedic studies are listed in Table 3. The animal models cited in the table are selected representatives with the authors' preference. Most of them are recent publications and are not necessarily the original models. Also, they are not inclusive.

TABLE 3
Author's Preferences of Animal Models for Common Orthopaedic Studies

System & Studies	Animal	Model	First author, year ^{Ref.}
Bone			
Osteogenesis in soft tissue	Nude mice/Rat	Subcutaneous pocket on the back	Hara 1996 ⁸⁰ /Nathan 1988 ¹⁰³
Fracture or osteotomy	Rat/Rabbit	Intramuscular implantation	Aspenberg 1989 ¹⁰⁴ /Ripamonti 1996 ¹⁰⁵
	Rat	Tibial/femoral fracture	Bak 1988 ²³ /An 1994 ²⁴ /Bonnarens 1984 ²⁵
	Rabbit	Radial/tibial fracture or osteotomy	Bruce 1987 ¹⁰⁶ /Braten 1990 ¹⁰⁷ /Murray 1996 ¹⁰⁸
	Dog	Radial fracture	Bellah 1987 ⁴⁴ /Glennon 1994 ⁴⁵
Bone defect or bone graft	Rabbit/Rat	Femoral condyle osteotomy	An 1997 ¹⁰⁹
	Rabbit/Dog/Rat	Calvarial defect	Schmitz 1986 ¹² /Ashby 1996 ¹³ /Kobayashi 1995 ²⁷ /Sweeney 1995 ²⁸
	Dog/Sheep/Rat	Radial defect	Niedzwiedzki 1993 ¹⁰ /Nyman 1995 ¹¹ /Johnson 1996 ⁴⁶ /Solheim 1992 ²⁶
Bone ingrowth	Dog/Goat	Large femoral defect	de Pablos 1994 ¹¹⁰ /Ehrnberg 1993 ¹¹¹ /Puelacher 1996 ¹¹²
		Transcortical plug	Cameron 1976 ³⁷ /Boby 1980 ¹¹³ /Verheyen ¹¹⁴
	Rabbit	Femoral condyle plug	Søbelle 1991 ¹¹⁵
		Distal femur plug (transverse)	Friedman 1995 ²⁰ , 1996a ²¹ , 1996b ¹⁶
		Distal femur plug (longitudinal)	Feighan 1995 ³
	Rat	Tibial/femoral intramedullary nail	Ducheyne 1992 ²⁹ /Hazen 1993 ³⁰
Bioabsorbable implant	Rat/Rabbit	Subcutaneous implantation	Pistner 1993 ¹¹⁶ /Törmälä 1991 ¹¹⁷
	Rabbit	Femoral/tibial osteotomy	Bostman 1992 ¹¹⁸ /Matsusue 1991 ¹¹⁹
	Dog	Femoral condyle osteotomy	An 1997 ¹⁰⁹
		Femoral diaphyseal osteotomy	An 1997 ¹⁰⁹ /Leggon 1988 ²⁰
Osteonecrosis	Dog	Femoral head/freezing	Malizos 1993 ⁵³
	Rabbit	Femoral head, steroid/Shwartzman reaction	Matsui 1992 ⁴ /Yamamoto 1995 ⁵
	Pig	Femoral head, posttraumatic	Seller 1996 ⁸⁸
	Rat	Femoral head, hypertensive	Kataoka 1992 ¹²¹
Osteoporosis or osteopenia	Rat/Primate	Ovariectomy	Cesnjaj 1991 ¹³ /Thompson 1995 ³⁴ /Kimmel 1996 ²²
Bone lengthening	Rabbit/Dog/Goat	Tibia	Nakamura 1995 ¹²² /Daniel 1994 ¹²³ /Lin 1996 ¹²⁴
	Rat	Tibia (Ilizarov type)	Aronson 1997 ¹²⁵
Inhibition of physal growth	Rabbit	Upper tibial physal stapling, ablation	Ross 1997 ¹²⁶
Prosthetic infection	Rabbit	Femoral head/knee replacement	Southwood 1985 ¹²⁷ /Blomgren 1981 ¹²⁸ /Belmatoug 1996 ¹²⁹
		Femoral condyle metal plug	An 1997 ¹⁹ /Isiklar 1996 ¹³⁰
	Dog	Femoral/tibial intramedullary nailing	Petty 1985 ⁴⁹ /1988 ⁵⁰ /Isiklar 1993 ¹³¹

TABLE 3 (continued)
Author's Preferences of Animal Models for Common Orthopaedic Studies

System & Studies	Animal	Model	First author, year ^{Ref.}
Osteomyelitis	Rabbit/Rat Dog	Tibia Tibia/femur	Scheman 1941 ¹³² /Mayberry-Carson 1992 ¹⁷ /Rissing 198 ⁵³ /Spagnolo 1993 ³⁵ Fitzgerald 198 ³⁵ /Philipov 1995 ¹³³
Joint			
Septic arthritis	Mouse/Rat	Multiarthritis, hematogenous <i>S. aureus</i>	Bremell 1991 ¹³⁴ /Bremell 1994 ³²
Osteoarthritis	Rabbit Dog/Rabbit Guinea pig	Knee, intraarticular injection of <i>S. aureus</i> Knee Knee	Stricker 1996 ¹⁸ Moskowitz 199 ²⁶ de Bri 1995 ⁹⁵ /Watson 1996 ⁹⁶
Inflammatory arthritis	Rabbit Rat	Knee injection of carrageenan/antigen Collagen-induced arthritis	Bogoch 1988 ¹⁴ /Kang 1997 ¹⁵ /Beesley 1992 ¹³⁵ Knoetzer 1995 ¹³⁶
Joint replacement	Dog Rabbit Rat	Total hip replacement Femoral head replacement Simultaneous air pouch	Kraemer 1995 ⁵² /Finkelstein 1995 ¹³⁷ /Dowd 1995 ¹³⁸ Southwood 1985 ¹²⁷ Gelb 1994 ³⁷ /Naidu 1996 ¹³⁹
Biomaterial debris	Rabbit Dog	Intraarticular administration Proximal tibia implantation of debris Total hip replacement	Lewis 1995 ³⁸ Goeman 1991 ¹⁴⁰ /1994 ¹⁴¹ Boby 1995 ¹⁴² /Kraemer 1995 ⁵²
Cartilage			
Subcutaneous chondrogenesis	Nude mice	Subcutaneous pocket on the back	Paige 1996 ⁸ /Fujisatao 1996 ⁵²
Articular cartilage defect	Rabbit/Dog	Distal femoral joint defect	Freed 1994 ⁷ /Kandel 1995 ⁹ /Brittberg 1996 ⁹ /Shortkroff 1996 ¹⁴³
Meniscus repair and grafting	Rabbit/Dog/Goat Rabbit/Dog/Sheep	Repair Grafting or reconstruction	Huang 1989 ¹⁴⁴ /Armoczek 1994 ⁵⁴ /Miller 1995 ¹⁴⁵ Messner 1994 ¹⁴⁶ /de Groot 1996 ⁵⁵ /Edwards 1996 ⁷⁰
Ligament and tendon			
MCL repair	Rabbit/Rat	Complete or partial laceration of CML	Frank 1995 ¹⁴⁷ /Schreck 1995 ¹⁴⁸ /Litke 1994 ¹⁴⁹ /Batten 1996 ¹⁵⁰
MCL reconstruction	Rabbit	Autograft or xenograft	Milthorpe 1994 ¹⁵¹ /King 1995 ¹⁵²
ACL reconstruction	Rabbit/Goat/Dog	ACL reconstruction	Armoczek 1990 ²
Artificial ACL anchor	Goat	ACL reconstruction	Young 1995 ³⁶
Tendon repair	Chicken/Rabbit	Laceration of flexor profundus tendons	Kugota 1996 ¹⁰¹

Soft tissues

Biomaterial biocompatibility	Rat/Mice	Intramuscular implant	McNamara 1981 ⁴⁹ /McGeachie 1992 ¹⁵³
Small diameter artificial vessel	Rat/Dog	Subcutaneous implant	An 1997 ³⁹ /Picha 1996 ¹⁵⁴ /Hunt 1996 ¹⁵⁵ /Campbell 1989 ¹⁵⁶
Nerve repair/graft	Rat	Abdominal aorta	Bartels 1988 ⁴¹ /Okoshi 1993 ¹⁵⁷
	Rabbit	Carotid artery, infrarenal aorta	Cassel 1989 ¹⁵⁸ /Greisler 1991 ¹⁵⁹
	Dog	Femoral artery/carotid artery/aorta	Matsuimoto 1973 ¹⁶⁰ /Sandusky 1995 ¹⁶¹ /Kito 1996 ¹⁶²
	Rat/Rabbit	Ischiatic/sciatic nerve defect/grafting	Hall 1994 ⁴² /Giandino 1995 ⁴³ /Amillo 1995 ¹⁶³
Spine			
Spinal instability	Rabbit/Pig	Lumbar spine	Stokes 1989 ¹⁶⁴ /Kaigle 1995 ¹⁶⁵
Spinal fusion	Rabbit/Dog/Goat	Lumbar spine	Boden 1995 ¹⁶⁶ /Sandhu 1997 ¹⁶⁷ /Brantigan 1994 ¹⁶⁸
Spinal cord compression	Mouse/Rabbit	Lumbar spine	Miyamoto 1992 ¹⁶⁹ /Saito 1992 ¹⁷⁰
Verteb. column graft/replacement	Dog	Thoracic spine defects	Olson 1991 ¹⁷¹
Interverteb. disc graft/replacement	Dog	Lumbar spine	Frick 1994 ¹⁷² /Matsuzaki 1996 ¹⁷³
Scoliosis	Chicken/Rabbit	Pinelectomy/rib-growth stimulation	Kanemura 1997 ¹⁰⁰ /Agadir 1988 ¹⁷⁴

E. FDA GUIDELINES

For certain animal models, the animal selection is relatively easy since there are guidelines established by professional authorities. In the United States, guidelines have been established by the FDA for certain preclinical and clinical studies such as preclinical testing of new drugs. For example, the recent *FDA Guidelines for Preclinical and Clinical Evaluation of Agents Used in the Treatment or Prevention of Postmenopausal Osteoporosis (1994)* delineate specific preclinical models to demonstrate the efficacy and safety of new, potential agents for osteoporosis therapy.¹⁷⁵ The guidelines recommend that agents be evaluated in two animal species, including a ovariectomized rodent model (rat) and a second non-rodent model (large remodeling animals, dogs, or preferably ovariectomized primates). Although there are some controversies about individual guidelines,³⁴ most of the guidelines are scientifically sound.

III. CONCLUDING REMARKS

The selection of an animal model sounds easy, but it is not. Many factors have to be considered before the decision is made, including the appropriateness of the model to the human condition, the available background data of the animal, the availability, housing requirement, cost, and the ease of experimental manipulation of the animal, as well as the ethical implications of using research animals.

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4 Surgical Facilities, Peri-Operative Care, Anesthesia, and Surgical Techniques

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I. INTRODUCTION

Successful outcomes in the use of animal models in orthopaedic research are best achieved by an interdisciplinary approach among researchers, surgeons, veterinarians, and animal care staff. This approach also generally results in optimization of the ethical and welfare aspects for the animals involved. A team approach involves coordination of all facets of an animal protocol, beginning with preoperative planning to identify any special needs related to the anesthetic or operative regimens as well as requirements for postoperative care. Careful protocol preparation and IACUC review will necessitate that these protocol issues be described; however, some degree of firsthand interaction among key personnel can contribute greatly to the smooth initiation of a new study or the successful modification of one with unanticipated experimental difficulties. Each of the various professional and technical backgrounds have different but unique perspectives and areas of expertise to bring to bear on any given project and those different areas of proficiency should be used to advantage.

As in any survival surgical procedure, but especially in orthopaedic surgery, meticulous attention to aseptic technique and appropriate tissue handling is necessary to minimize the possibility of infection as well as the experimental variables associated with exaggerated tissue responses to trauma. In many cases it may be necessary for the MD surgeon to become familiar with species-specific anatomical variations for optimal technical results. In addition, MDs may need to be educated regarding the species-specific differences that dictate which anesthetic regimens are appropriate as well as which postoperative medications and practices are suitable. Guidelines for training of personnel who perform surgery on research animals have been published and should be consulted.^{1,2}

The design of the surgical and support areas should be such that all aspects of providing appropriate medical and surgical practices are facilitated. Facilities and equipment should be suitable for the species of animals utilized and the complexity of the procedures performed. Regulatory and legal guidelines must also be met. This chapter will provide an overview of the issues related to animal anesthesia and surgery.

II. SURGICAL FACILITIES AND EQUIPMENT

The principal issue regarding surgical facilities in the Animal Welfare Act is more of a programmatic issue rather than an engineering standard. The USDA regulations state that major survival surgical procedures in nonrodent species must be performed in a dedicated facility which is maintained under aseptic conditions. Aseptic procedures include the use of surgical attire, sterile instruments and aseptic techniques. Minor operative procedures and survival surgery in nonrodent species do not require a dedicated facility but must still be performed using aseptic procedures.³

The guide makes similar programmatic recommendations but is more specific regarding what are considered suitable design features for aseptic surgical facilities.⁴ Emphasis is placed on the integration of different functional areas and features that help minimize contamination. Design features that are described are general recommendations. What is important is that the facility be appropriate for the species used, the complexity of the procedures, and the magnitude of the surgical program.

The guide recommends the following functional elements for most survival surgical facilities: surgical support, animal preparation area, surgeon's scrub area, operating rooms, and postoperative recovery. In some cases, with particularly low volume programs using only a few species, it may be appropriate to combine certain functional areas such as the animal preparation and postoperative recovery areas. Some factors which would influence the suitability of this arrangement would include whether compatible species were recovered simultaneously and if noise could be minimized for animals recovering from anesthesia. In general, the guide recommends that the entire surgical area be physically separate from the rest of the animal facility, but that careful consideration be given to its physical relationship to animal housing areas, radiology facilities, diagnostic laboratories, and office areas.

Design features to reduce the potential for contamination should be utilized. Use of solid, nonporous materials for interior surfaces and minimizing the amount of fixed equipment in the operating room facilitate sanitization. Minimizing traffic flow in the operating rooms and appropriate ventilation systems are also critical.

Other design features should include availability of adequate lighting, scavenging of waste anesthetic gases, and sufficient electrical outlets for support equipment. The size of the operating room should be determined by which species are used, the amount of support equipment necessary, and the number of staff involved in procedures. For a more detailed description of issues related to the design of surgical facilities, the reader is referred to Ruys.⁵

Practices should be in place to maintain the operating room as free from contaminants as possible. All floors, lights and furniture should be wiped down with a suitable disinfectant prior to and after each use, as well as at the end of the day. Personnel should don surgical scrub suits, caps, masks, and shoe covers prior to entering the operating room.

All surgical instruments and bioimplants must be sterilized either by autoclaving or ethylene oxide sterilization. Instrument packs should be prepared to allow adequate air removal and steam penetration of the package during autoclaving. They should be double wrapped and external chemical indicators should be used on all packs, regardless of sterilization method. Efficacy of specific steam sterilization cycles should be monitored using biological indicators.

The type of surgical procedure will dictate the specific instrument needs, however, purchase of high quality surgical instruments will be most economical in the long run. Cleaning and decontamination of instruments should be performed as soon as possible after use since effective

decontamination is impaired if debris is allowed to dry on surfaces. Instruments should be dry prior to wrapping in order to prevent the potential for wet instrument packs after autoclaving. Guidelines for sterilization procedures have been published and should be consulted for appropriate practices.⁶

Proximity of the operating suite to radiology facilities is important for orthopaedic procedures. Radiographic equipment should be able to accommodate species of diverse sizes and be periodically maintained and calibrated. In addition, the room should be spacious enough to accommodate a portable anesthetic machine and a gurney for taking intra- or immediate postoperative films. Automatic processors are convenient for programs that perform large numbers of radiographs. It may also be useful to have view boxes mounted in the operating room in a location easily viewed by the surgeon.

A well-appointed surgical suite will have an array of equipment related to the maintenance and monitoring of anesthesia in addition to equipment used by the surgeon. Gas anesthesia machines, ventilators and ECG monitoring devices are routinely used for most nonrodent species undergoing anesthesia. Similarly, circulating water blankets and electronic temperature probes are also routinely employed. Blood pressure monitoring by direct or indirect methods may be indicated for particularly lengthy procedures. Infusion pumps provide accurate delivery of balanced electrolyte solutions and may also be utilized for anesthetic protocols that involve continuous infusions of parenteral agents. Electrocautery and suction may also greatly facilitate surgical manipulations. More specialized equipment may be necessary for projects that involve arthroscopic techniques.

III. PREOPERATIVE CARE

Preoperative care procedures and health monitoring will vary with the species utilized and the requirements of the experiment. All protocols should require the use of healthy and relatively young animals. Knowledge of the times of closure of various growth plates for the species utilized is often a determining factor for selecting the age range for the species to be ordered for orthopaedic research.⁷⁻¹⁰

Reputable commercial vendors that supply specific pathogen free animals should be used as the source for rodents and rabbits. These suppliers routinely perform intensive health monitoring to document quality assurance and freedom from endogenous pathogens in their animals. Presence of endogenous viral or bacterial pathogens can be an important source of experimental variation in terms of an animal's ability to undergo anesthesia, initiate and sustain normal tissue response to surgery, as well being free of a reservoir of bacteria that can potentially seed any bioimplants. Vendor health monitoring reports can be supplemented with in-house laboratory tests if vendor surveillance is desirable, however, generally no preoperative work-up is performed prior to surgery for these species.

Because of their extremely high metabolic rate, it is not necessary or desirable to withhold food or water from rodents preoperatively. An overnight fast is recommended for rabbits; however, they should be allowed free access to water until just prior to surgery. For these species, to assure a good surgical outcome, emphasis should be placed on intraoperative support, appropriate anesthetic regimens, limiting operating time to as brief a period as possible and providing suitable analgesics postoperatively when necessary.

Dogs expressly bred for research with comprehensive health profiles can be obtained from commercial vendors and are the most expensive to purchase. Alternatively, dogs can be obtained from USDA licensed Class B dealers or, in some areas, from local animal shelters. Dogs purchased from Class B dealers, while not purpose-bred for research, are healthy animals released for sale after undergoing a health-screening and "conditioning" regimen. If animals are available from local shelters, their low purchase price is frequently offset or negated by their uncertain or poor health status. These animals must undergo a comprehensive health-screening and conditioning regimen before they can be released for research protocols.

Sources of agricultural species used in research are more variable. Swine and some ruminant species can be obtained from commercial sources. However, it is also common to purchase farm animals directly from nearby farms. Farm source animals, particularly some ruminant species, should be routinely tested for species specific zoonotic diseases.

As a minimum, routine preoperative testing of dogs and other large animal species generally involves documentation that the animals are parasite-free and that they have a normal hematological profile. Other tests, such as blood chemistry or coagulation profiles, should be performed based on study requirements. Preoperatively large animals should be fasted overnight, although access to water should be allowed.

IV. ANESTHESIA AND ANALGESIA

The diverse species of laboratory animals used in orthopaedic research precludes a detailed discussion of all of the anesthetic agents that can be used for these protocols. Species specific physiologic requirements and biological parameters should be discussed with a veterinarian prior to initiating the protocol. Principles of anesthesia and general anesthetic recommendations will be discussed in this selection. Veterinary textbooks on anesthesia and analgesia should be used as a resource if more specific information is required.¹¹⁻¹⁶

A. PRINCIPLES OF ANESTHESIA

Orthopaedic procedures are generally invasive major surgeries requiring deep surgical anesthesia. The anesthetic protocol should provide anesthesia, analgesia and muscle relaxation while maintaining homeostasis. The physiologic effects of the anesthetic protocol should be carefully considered, especially if physiologic measurements are to be made while under anesthesia.^{14,17,18} The selection of the protocol will also depend upon the equipment available and the expertise of the personnel. In general, inhalant anesthesia is the preferred method for general anesthesia in most species. Dosages of agents for species likely to be used in research are in the charts, however, the reader should also refer to the text for discussion of indications and contraindications. Postoperative analgesia is indicated for orthopaedic protocols unless it must be withheld for scientific reasons.

B. PREOPERATIVE AGENTS

Preoperative agents are used to sedate the animal to prevent anxiety, to lower the dosage of general anesthetics administered and to prevent undesirable physiologic reactions, such as vagal stimulation. Preoperative agents fall into the classes of anticholinergics, tranquilizers, dissociative agents and α -adrenergic agonists and antagonists.¹¹⁻¹⁶

Anticholinergic agents such as atropine are useful to prevent bradycardia during intubation and to dry bronchiole secretions through its vagolytic action. Glycopyrrolate may be used as an atropine substitute. The agents are generally administered 5–15 min. prior to induction under general anesthesia, usually in combination with other preanesthetic agents. Atropine will induce a transient tachycardia and would be contraindicated if that effect is undesirable.¹¹⁻¹⁶

Tranquilizers are used to sedate animals and to reduce the dosage of general anesthetic agents. They are generally administered 15–30 min. prior to general anesthesia. The phenothiazine derivative tranquilizers are the most common ones used in veterinary anesthesia, in particular, acepromazine. Other agents such as promazine and chlorpromazine may induce undesirable side effects in some species. The phenothiazine derivatives are mild α -adrenergic blockers and also cause peripheral vasodilation. Their effects generally last approximately 8–12 hours.¹¹⁻¹⁶

Benzodiazepine tranquilizers include diazepam and midazolam. These agents provide good sedation with minimal cardiovascular effects. Midazolam is a water soluble agent which is more potent and shorter acting than diazepam.¹⁹ When used as a sole agent it has a duration of action of

approximately 20 min. Diazepam is relatively long acting with effects of 4–8 hours in most species. Diazepam is effective to counteract seizure activity. These agents may be combined with other agents to induce surgical anesthesia for minor procedures.

Dissociative agents include ketamine and the combination agent tiletamine-zolazepam (Telazol).²⁰ These agents may be used to induce animals into a state of dissociative anesthesia causing them to be unaware of their surroundings but with only mild analgesia. Animals will not have muscle relaxation and will have mild clonic/tonic types of muscular activity. These agents are extremely useful for chemical restraint of approximately 20 min. in most species. They may be used as part of a general anesthesia protocol when combined with other agents. Their use as general anesthetics is discussed under injectable agents. Ketamine causes a transient tachycardia with minimal hemodynamic effects. The cardiovascular effects of Telazol may be more pronounced in some species.^{11–16}

Alpha adrenergic agonists and antagonists include xylazine and medetomidine,²¹ which are the two most commonly used agents in this class. They are best utilized in combination with other agents to provide general anesthesia. Xylazine causes bradycardia, heart block, peripheral vasodilation and nausea in many species. These side effects can be counteracted with atropine. Medetomidine has less of the undesirable effects of xylazine. These agents have mild analgesic activity, which may be very transient in some species. They generally have activity for 20 min.^{11–16}

C. INJECTABLE ANESTHETIC PROTOCOLS

When injectable agents are utilized for general anesthesia, they should be administered as continuous iv infusions following induction. The use of infusion protocols enables the anesthetist to provide a stable level of physiologic effects instead of the unpredictable fluctuations in baseline activities that may occur when repeated bolus injections are administered. Combining agents also causes variations of the effects of the individual agents to occur depending upon the species and the dosages. Combinations may either potentiate or nullify the undesirable effects of some agents making it difficult to predict the physiologic effects of the protocol.^{14,16} Commonly recommended injectable anesthetic protocols are listed in Table 1.

Dissociative agents have been widely combined with other agents to induce anesthesia. As sole agents, they do not provide sufficient analgesia for general surgery. The two most commonly used agents are ketamine and the combination agent tiletamine/zolazepam (Telazol).²⁰ They are usually combined with tranquilizers or α -adrenergic agents. Commonly used combinations are: ketamine/acepromazine, ketamine/diazepam, ketamine/midazolam, ketamine/xylazine, ketamine/medetomidine, ketamine/Telazol, ketamine/Telazol/xylazine. None of these combinations can be recommended for all species and protocols. Their individual effects are very species dependent. Most of the combinations provide 20–30 min. of general anesthesia in most species, which is not sufficient for prolonged or highly invasive surgeries.^{11–16}

Barbiturate anesthesia is used in all species. However, it requires iv access for most species. They may be administered as an ip injection for rodents. The barbiturates are potent cardiorespiratory depressants which are dose dependent in their activity. In large animals, they are best administered as iv infusion protocols. Pentobarbital is relatively long acting in most animals and may provide 30–45 min. of anesthetic activity following a single iv injection. It requires hepatic metabolism and excretion for biodegradation. Thiobarbiturates, such as thiopental and thiamylal, are primarily excreted by the kidneys and have much shorter anesthetic times of 5–20 min. The dosage of barbiturates are reduced 1/3–1/2 by administration of tranquilizers or other preanesthetic agents.^{11–16}

Propofol is a steroidal anesthetic that must be administered by continuous iv infusion. The agent may be profoundly hypotensive in some species. It may be administered as a sole agent for general anesthesia in some species.^{14–16}

Other agents such as detomidine, etomidate and alpha chloralose are generally not reliable for general anesthesia in most species.^{14–16} They may be indicated as part of a general anesthetic

TABLE 1
Commonly Recommended Injectable Anesthetic Protocols*

Animal	Agent ^{Ref.}	Dosage	Route
Mouse or Rat	Fentanyl/fluanisone+midazolam ^{†11}	10 ml/kg for mice, 2.7 ml/kg for rats	IP
	Pentobarbital ^{14,15,16,17}	40–50 mg/kg	IP
	Ketamine/xylazine ^{11,14,15,16,17}	100 mg/kg + 10 mg/kg	IP
Guinea Pig	Fentanyl/fluanisone+midazolam ^{†11}	8 ml/kg	IP
	Pentobarbital ^{11,14,15,16,17}	35–40 mg/kg	IP
	Ketamine/xylazine ^{11,14,15,16,17}	50 mg/kg + 2 mg/kg	IP
Rabbit	Fentanyl/fluanisone+midazolam ^{†11}	0.3 ml/kg (IM) + 2 ml/kg (IV)	IM+IV
	Pentobarbital ^{11,14,15,16,18}	40–45 mg/kg	IV
	Ketamine/xylazine ^{11,14,15,16,18}	35 mg/kg + 5 mg/kg	IM
	Ketamine/medetomidine ^{11,15,16,18}	25 mg/kg + 0.5 mg/kg	IM
	Ketamine/acepromazine ^{11,14,15,16,18}	50 mg/kg + 1 mg/kg	IM
Cat	Ketamine/acepromazine ^{11,3,5,14,15,19}	10–20 mg/kg + 1 mg/kg	IM
	Ketamine/xylazine ^{11,3,5,14,15}	15 mg/kg + 1 mg/kg	IM
	Ketamine/medetomidine ^{11,15}	7 mg/kg + 0.08 mg/kg	IM
	Tiletamine/zolazepam ¹⁵	6–12 mg/kg	IM
	Pentobarbital ^{11,14,15,19}	20–30 mg/kg	IV
Dog	Thiopental ^{11,14,15,19}	8–12 mg/kg	IV
	Fentanyl/fluanisone ¹¹	0.1–0.2 mg/kg	IM
	Ketamine/midazolam ^{11,15}	10 mg/kg + 0.5 mg/kg	IM
	Tiletamine/zolazepam ^{14,15,19}	6–12 mg/kg	IM
	Pentobarbital ^{11,14,15}	20–30 mg/kg	IV
Goat or sheep	Thiopental ^{11,14,15}	8–12 mg/kg	IV
	Ketamine/xylazine ^{12,14,15}	2.2–7.5 mg/kg+0.1 mg/kg	IV
	Ketamine/medetomidine ^{12,15}	0.5 mg/kg + 0.02 mg/kg	IV
	Pentobarbital ^{12,15}	20–30 mg/kg	IV
Swine	Thiopental ^{12,14,15}	25 mg/kg	IV
	Ketamine/acepromazine ^{11,12,14,15,16}	33 mg/kg + 1.1 mg/kg	IM
	Ketamine/medetomidine ^{14,15,16,21}	10 mg/kg + 0.2 mg/kg	IM
	Tiletamine/zolazepam ^{14,15,16}	2–8 mg/kg	IM
	Pentobarbital ^{11,12,14,15}	20–40 mg/kg	IV
Baboon	Thiopental ^{6,11,12,14,15,16}	6.6–25 mg/kg	IV
	Ketamine ^{14,15,24}	5–10 mg/kg	IM
Macaques	Ketamine/diazepam ^{14,15,24}	10 mg/kg + 0.2 mg/kg	IM
	Ketamine ^{14,15,24}	5–20 mg/kg	IM
	Ketamine/xylazine ^{14,15,24}	10 mg/kg + 0.25 mg/kg	IM

* These injectable agents provide approximately 20–30 minutes of anesthesia in most species. For prolonged procedures it is best to maintain general anesthesia after induction with an inhalational anesthetic (see text).

† Mixture of 1 part fentanyl/fluanisone + 2 parts water for injection + 1 part midazolam.

protocol in special circumstances. The combination agent fentanyl/fluanisone (Hypnorm) is commonly used in rodents.¹¹

D. INHALATIONAL ANESTHESIA

Inhalational anesthesia should be the primary choice for general anesthesia unless it is contraindicated by the scientific protocol or the inability of laboratory personnel to administer it properly. The most commonly used inhalant anesthetics in veterinary anesthesia are isoflurane, halothane and methoxyflurane. Other newer agents include desflurane, sevoflurane and enflurane.

Older agents such as ether and chloroform should not be considered for general anesthesia. The agent that has the most widely indicated applications is isoflurane. It provides surgical anesthetic levels at concentrations ranging from 0.5-2.0% in most species. Nitrous oxide does not provide sufficient analgesia as a sole agent for general anesthesia in animals and should only be used in combination with other agents to reduce the level of the inhalant required.^{11-16,22}

Nitrous oxide, halothane and methoxyflurane have biosafety considerations when used in the laboratory. In susceptible individuals, they may cause hepatic and renal complications. All inhalant anesthetics, and especially these agents, require gas scavenging systems.¹¹⁻¹⁶

All inhalational anesthetics are best utilized with equipment designed for their delivery, including a vaporizer in a closed or semi-closed system. In rodents they are sometimes delivered as open agents on cotton balls, but this use should be limited to fume hoods or the equivalent. Equipment and hoses should be checked for leaks and soda lime canisters for absorption of carbon dioxide should be routinely cleaned and changed.

Inhalant agents are delivered in oxygen, nitrous oxide, air or a combination after flowing the delivery gas through a vaporizer. Flow rates for most species range from 10-15 ml/kg. The number of respirations required per minute is highly species and age dependent. If positive ventilation is used, most species require 18-22 cm H₂O airway pressure. The most common protocols for general anesthesia are isoflurane 0.5-2% delivered in oxygen or oxygen:nitrous oxide 2:1.¹¹⁻¹⁶

E. ANALGESIA

Postoperative analgesia should be required for all orthopaedic protocols unless a specific scientific justification exists for withholding them. Intraoperative or preoperative administration of these agents has been shown to reduce the pain reflex and to reduce the postoperative recovery times of some species. Animals should at least receive the first injection of an analgesic prior to recovering from general anesthesia. The length of administration depends upon the clinical condition of the animal and requires professional judgment.²³

The most commonly used agents in veterinary analgesia are the opioids. Specifically, buprenorphine and butorphanol are the most commonly used agents for most species. Some opioids, such as morphine, are associated with a high incidence of untoward reactions in some species.^{14,16,17,23,24} Common analgesics are listed in Table 2.

Nonsteroidal antiinflammatory drugs (NSAIDs) may be used in combination with opioids to enhance analgesia and antiinflammatory activity. The most widely used agent in animals is phenylbutazone. It does not provide sufficient analgesia postoperatively for major surgery, however, it may be used to reduce the dosage of opioids. Other agents, such as aspirin and acetaminophen, are less likely to be potent enough to be used in orthopaedic surgery and have hepatic and renal toxicity in some species. Newer NSAID agents, such as ketaprophen, may be sufficient to provide postoperative analgesia in some species. Their use in many species has not been validated.¹⁴⁻¹⁶

Injections of local anesthetics may also be used to enhance analgesia. They may be given as dorsal nerve root injections to provide regional analgesia or as infiltrations along the incision line.¹⁴⁻¹⁶

V. SURGICAL TECHNIQUE

Strict adherence to aseptic procedures and principles of careful tissue handling are necessary for the evaluation of biomaterials. The goal of the surgeon should be to minimize all factors that have the potential to impede normal wound healing, thereby making the interpretation of data most meaningful by minimizing experimental variability. The precepts that are outlined below should be applied to all species undergoing orthopaedic procedures.

Preparation of the animal involves decontamination of the incision site and enough of the surrounding surgical area to prevent wound contamination. Hair removal and initial skin cleansing

TABLE 2
Commonly Recommended Analgesics

Agent	Animal ^{Ref.}	Dosage (mg/kg)	Route	Duration of Action (hrs)
Buprenorphine	Mouse ^{11,14,23,24,25}	0.5–2.0	SC	12
	Rat ^{11,14,23,24,25}	0.1–0.5	SC	12
	Guinea Pig ^{11,14,23,24,25}	0.5	SC	8–12
	Rabbit ^{11,14,23,24,25}	0.02–0.05	SC	8–12
	Cat ^{11,13,14,15}	0.005–0.01	SC	12
	Dog ^{11,13,14,15}	0.01–0.02	SC	12
	Sheep ^{11,12,13,14,15}	0.005–0.01	IM, SC	6
	Goat ^{11,12,13,14,15}	0.005	IM, SC	6–12
	Swine ^{11,12,13,14,15,16}	0.05–0.1	IM	12
	Primates ^{11,12,13,14,15,24}	0.01	IM	6–8
	Mouse ^{11,14,23,24,25}	1–5	SC	4–6
Butorphanol	Rat ^{11,14,23,24,25}	2	SC	6
	Rabbit ^{11,14,23,24,25}	0.1–0.5	SC	6
	Cat ^{11,13,14,15}	0.4	SC	6
	Dog ^{11,13,14,15}	0.2–0.4	SC	6
	Sheep ^{11,12,13,14,15,20}	0.5	SC, IM	4–6
	Swine ^{11,12,13,14,15,16}	0.1–0.3	SC, IM	4–6
	Mouse ^{14,15}	30	PO	12
Phenylbutazone	Rat ^{14,15}	20	PO	12
	Guinea Pig ^{14,15,17}	40	PO	12
	Cat ^{14,15}	10	PO	12
	Dog ^{14,15}	6–22	PO	8
	Goat or sheep ^{12,14,15}	4–8	PO	12
	Swine ^{14,15,16}	10–20	PO	12
	Primate ^{14,15,24}	50	PO	12
	Dog ¹⁴	1–1.5	PO	6
Ketorolac	Swine ¹⁶	1	IM, PO	12
	Primates ²⁴	1	IM	12

should be performed outside the operating suite in the case of rabbits and large animals and in a site separate from the operating area for rodents. Hair should be removed as close to the skin as possible while simultaneously maintaining the integrity of the epithelium. Loose hair should be thoroughly removed from the surgical area followed by application of a surgical antiseptic beginning over the incision site and working outward to avoid contamination from the periphery.

Following transport to the operating room or table, a “sterile” scrub is performed prior to draping. Three successive scrubs with a surgical disinfectant are performed using sterile gauze sponges, sponge forceps and surgical gloves using the same outward pattern. An application of 70% alcohol follows a suitable contact time for the disinfectant used, then the entire area is dried. It is then preferable to apply a plastic, incisable drape over the entire area just prepared. This type of drape is a better bacterial barrier than cloth drapes because it is impermeable to fluids and prevents wicking of moisture from the periphery into the incision.

Draping proceeds by closely surrounding the incisional area with four drapes followed by covering the entire animal and back table with one large drape. For large animals separation of the sterile field from the anesthetist at the head of the table is accomplished by using two IV poles positioned on opposite sides of the table to suspend the large drape with towel clamps. The end result, even for rodents and rabbits, should be the formation of one entire sterile field that includes the completely draped animal, operating table, and adjacent back table with instruments. This

method of draping greatly facilitates the maintenance of a sterile field and instruments during surgery.

Use of good surgical technique is critical to produce the minimal amount of tissue damage necessary to the procedure. Gentle tissue handling, meticulous hemostasis, and closure of dead space all contribute to minimizing the intensity and duration of the inflammatory response and preventing hematoma or seroma formation that favor bacterial growth. Incisional margins should be carefully apposed to avoid excessive tension which can create irritation postoperatively and result in self-mutilation. Suture material should be selected to minimize the inflammatory response, making synthetic absorbable suture materials preferable to catgut and silk. In species with an adequate amount of subcutaneous tissue, skin closure is best accomplished with a buried suture line using a subcuticular pattern. Monofilament nylon or stainless steel wound clips can be used for skin closure of rodents; however, rodents may succeed in chewing out sutures or staples if tissue irritation occurs. Routine wound care for many procedures usually only requires observation of the incisional area for normal healing. Veterinary surgical textbooks should be consulted for species-specific techniques and practices.^{16,25–29}

VI. POST-OPERATIVE CARE

The postoperative period can be divided into two important phases, recovery from anesthesia and the period of tissue healing. All species should be carefully and frequently observed during recovery from anesthesia, which is characterized by presence of normal vital signs and return of righting reflexes. Intensity of postoperative monitoring will vary with the species; however, return to normothermia and attention to cardiorespiratory function are important in all species. Circulating water jackets can be safely used in all species to maintain body temperature without running the risk of tissue injury. Heat lamps and heating pads can also be used but in such a way as to prevent overheating or tissue damage. Intraperitoneal administration of warmed electrolyte solutions is an easy method of aiding rewarming and providing cardiovascular support to rodents. For rodents monitoring respiratory rate and pattern as well as mucous membrane color is advisable. Rodents should not be allowed to recover on wood shavings since they can occlude the nose and stick to the mouth and eyes. Soft cloths or disposable pads should be used instead and can aid in maintaining body temperature.

Monitoring vital signs, mucous membrane color, and surgical incisions at regular intervals should be routine for rabbits and large animals. ECG monitoring and use of pulse oximetry is also useful, but may not be necessary for all procedures. In addition to routine monitoring during postanesthetic recovery, resources should also be available to handle potential complications. This generally involves support of the cardiorespiratory system by the administration of fluids and emergency drugs as well as providing airway support and supplemental oxygen.

For many procedures, animals may be returned to their home cages following anesthetic recovery. However, in some cases it may be necessary to rely on cage confinement to limit activity for a certain period of time postoperatively. These situations require the use of professional judgment and should be worked out in advance among the researcher, veterinarian, and animal care staff. Postoperative monitoring should be performed at least once daily for a specified time frame and include measurement of vital signs; assessment for resumption of normal physiologic functions, species-specific behavior and activity levels; and healing of surgical incisions. In addition, a careful assessment should be made regarding the degree of postoperative pain and distress and the need for analgesia. This topic has received detailed discussion elsewhere to which the reader is referred for further information.^{30,31} Other precepts of good nursing care should be followed and include offering palatable foods to stimulate appetite, use of bandaging to provide padding for sore extremities, and providing a comfortable, quiet environment.

For orthopaedic procedures, it is especially important to evaluate the animal's ability to ambulate and perform normal body movements during the immediate postoperative period and at intervals

until study completion. Animals with musculoskeletal pain that cannot be adequately controlled with analgesics or animals that develop clinical musculoskeletal signs should be routinely radiographed, if necessary, under anesthesia in order to obtain diagnostic films. Animals with uncontrollable pain or distress should be euthanized.

For clinical diagnostic reasons as well as to meet certain study requirements, it is also necessary to have the means to perform various microbiological, hematological, and clinical chemistry tests. Meaningful information can only be obtained if appropriate procedures are followed during sample collection, processing, and transport. If in-house laboratory facilities are limited or unavailable, arrangements should be made in advance with commercial laboratories that can competently handle veterinary specimens.

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5 Euthanasia and Necropsy

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I. INTRODUCTION

The term *euthanasia* literally translated from the Greek means good death. Euthanasia techniques which result in rapid loss of consciousness with minimal stress or anxiety and are followed by cardiac and respiratory arrest and cessation of brain function are considered humane euthanasia methods.¹ Ideally, these processes rapidly disrupt all afferent sensory pathways from peripheral pain receptors as well as blocking central processing of these pathways in the thalamus, cerebral cortex and subcortical centers.¹

Investigators who need to euthanize an animal as part of a project must do so in compliance with relevant guidelines set forth in the *Public Health Service (PHS) Guide for the Humane Care and Use of Laboratory Animals*,² the *Recommendations of the AVMA Panel on Euthanasia (1993)*¹ in the United States, or the *Recommendations for Euthanasia of Experimental Animals (1997)* of the European Commission.³ Euthanasia may also become necessary as a way of alleviating pain or distress that cannot be eliminated by the use of analgesics. For animals on protocols where pain or distress may occur before such studies begin, it is essential that the researcher and the veterinarian agree on a point at which the animal will be terminated from the study for humane reasons and euthanized.⁴

It is essential that persons performing euthanasia be adequately trained in the methods of restraint of the animal as well as the technical skills required to perform the act so as to minimize pain, stress and anxiety in the animals. In the United States, the PHS guide requires that all methods of euthanasia proposed by researchers be reviewed by an Institutional Animal Care and Use Committee (IACUC).²

Once euthanasia has been done, it is essential that the animal be examined for loss of vital signs.^{1,4} If there is any uncertainty, one may also decapitate the animal or create a bilateral thoracotomy or pneumothorax or exsanguinate it to confirm death. It is preferable not to have other animals in the room where the euthanasia is being performed as powerful pheromones are frequently emitted by the animals being euthanized or sounds may be made that are distressful to other animals.^{1,2,4}

II. EUTHANASIA METHODS

The choice of methods is governed by many variables such as the species, age, physical condition, number of animals that need to be done at the same time, cost, availability of equipment or controlled substances, whether one needs tissues for metabolic or other studies that must be free of chemical residues and the skills of the person performing the act. Euthanasia methods are generally classified as physical techniques, chemical agents or inhaled gases.¹ Physical methods have a high potential for causing pain or stress prior to the loss of consciousness if not carried out properly. Therefore, all physical methods must be justified by researchers in their grants and protocols and they must be approved by the IACUC.² Physical method should only be performed by trained personnel so that it results in a quick and painless death with minimal stress to both the animal and the person performing it.^{1,2} Animals should always be handled as gently as possible to minimize anxiety and stress. As a rule, the non-explosive inhaled gases, carbon dioxide and barbiturates are preferred over the physical methods. A summary of the acceptable euthanasia agents for each species is summarized in Table 1.

TABLE 1
Euthanasia Methods Recommended by AVMA

Species	Decapitation	Cervical Dislocation	CO ₂ †	Barbiturate Overdose	Gas Anesthetic Overdose (in hood)	KCl or Exsanguination (under anesthesia)	T-61‡ (IV only)
Cat				A (IV, IP)	A	A	A
Chicken	A§	A	A	A (IV, IP)	A	A	A
Dog				A (IV, IP)	A	A	A
Goat				A (IV)		A	A
Guinea Pig	A		A	A (IV)	A	A	A
Hamster	A	A	A	A (IP)	A	A	A
Mouse	A	A	A	A (IP)	A	A	A
Primate				A (IV, IP)		A	
Rabbit	A	A*	A	A (IV, IP)	A	A	A
Rat	A	A*	A	A (IP)	A	A	A
Sheep				A (IV)		A	A
Swine				A (IV)		A	A

* Acceptable for immature animals only (rats & guinea pigs < 200g, rabbits , 1kg).¹

† Prolonged time is required for immature animals.

‡ Animals must be sedated prior to use.³

§ A = acceptable.

Animal caretakers, students and some researchers may find it psychologically disturbing to have to repeatedly euthanize animals.⁴⁻⁶ This is especially true when they have become emotionally attached to the animals. In such cases it is prudent to have someone else euthanize them. Skilled caretakers and technicians who can handle the animals compassionately at the time of euthanasia can do a lot toward preventing distress in the animals.

A. DECAPITATION OR CERVICAL DISLOCATION

Decapitation is acceptable for rodents weighing less than 200 grams and for small rabbits less than one kg.¹ The major advantage to this technique is that it provides a rapid loss of consciousness and is a quick way to obtain tissues with minimal metabolic artifacts or chemical residues.^{1,7} It is generally performed with a guillotine. Mikeska and Kiem⁸ believed that decapitation produces a powerful arousal stimulus and they demonstrated the presence of low voltage fast activity in the decapitated head for 13–14 sec. However, both they and Derr⁹ documented that the time required for the oxygen tension in the brain to drop low enough for the brain to lose consciousness from hypoxia is 2.7 seconds. In addition, Vanderwolf et al.¹⁰ have shown that this EEG activity is not indicative of a conscious state. It has been clearly shown that sectioning of the spinal cord at any level results in immediate loss of pain perception below the level of the section. Sensory fibers for the head and scalp enter the spinal cord at the levels of C2-C3.¹¹ It is for that reason that decapitation should be performed at the atlanto-occipital joint or at C1-C2. If done in this manner, decapitation is a humane method.^{1,10,12} The major disadvantages to this technique are that it is potentially dangerous to the person doing it and that it does require some handling and restraint of the animals.

Cervical dislocation is performed by placing a rod immediately behind the base of the skull and pressing down while at the same time pulling quickly on the tail or hindlimbs with the thumb and index finger until you feel the vertebrae separate. High cervical dislocation at the atlanto-occipital joint results in crushing and severing of the spinal cord.¹ Unlike decapitation, dislocation does not result in an almost immediate loss of consciousness as a result of loss of blood flow. Holson¹² believed that for this reason, this technique may cause more pain perception than decapitation. Death results from paralysis of respiration and subsequent anoxia. This technique is approved for rodents weighing less than 200 grams as well as poultry and rabbits less than 1 kg.¹ The person doing it must be very skilled at the technique in order to do it humanely. The advantages are the same as those for decapitation. This technique may result in pulmonary artifacts like blood in alveoli and vascular congestion.¹³

B. CARBON DIOXIDE

Carbon dioxide is a safe, inexpensive, non-flammable agent for euthanasia of rodents. Carbon dioxide can be delivered either in the form of dry ice or as a compressed gas in cylinders. If it is used in the form of dry ice, there must be no physical contact between the ice and the animal being euthanized.^{1,4} Carbon dioxide generated by any other source is not acceptable.¹⁻⁴ Neonatal animals are resistant to this agent so prolonged exposure is necessary or barbiturates could be used as they work well in neonates. Carbon dioxide results in rapid lowering of the pH of the blood and cerebrospinal fluid. Hypoxia and death are the result of direct depression of the cerebral cortex, subcortical structures and the respiratory center and cerebral vasodilatation as well as a direct effect on the myocardium.¹³ If a euthanasia chamber is used, care must be taken to avoid overcrowding.

Butler et al.¹⁴ have shown that carbon dioxide alters arachidonic acid metabolism and smooth muscle responses to acetylcholine. Carbon dioxide had also been documented to alter lymphoproliferation and cell mediated lympholysis.¹⁵ This has always been considered a painless agent. However, a recent study using human subjects has shown that high concentrations of carbon dioxide, 80–100%, can be noxious and painful to the nose and throat.¹⁶ This is believed to be due to the formation of carbonic acid on mucous membranes. The authors concluded that rats exposed to the

same concentrations may also experience pain and discomfort. They further suggested that carbon dioxide could still be considered a humane agent if the animals were not exposed to concentration greater than 70% until after they had become unconscious. Carbon dioxide does not alter the histologic integrity of most tissues including the brain but it may cause vascular congestion, pulmonary edema or microhemorrhages in the lungs of rodents.¹³

C. GAS ANESTHETIC OVERDOSE

Inhalant anesthetics include halothane, methoxyflurane, enflurane, isoflurane and ether. Ether is explosive, flammable and very irritating and for these reasons it is not recommended.¹⁷ Gas overdose is an excellent choice for small (<7 kg) animals where venipuncture is technically difficult and in chickens.¹ Halothane, methoxyflurane and isoflurane are nonflammable, nonexplosive and can be delivered either in a closed container like a closed chamber or bell jar or via a face mask. Care must be taken to ensure that there is an adequate supply of air inside the closed chamber and that the animal does not come in direct contact with the liquids as they are irritating. Halothane is the most soluble and most effective for inducing rapid loss of consciousness and, therefore, is the agent of choice.¹ Isoflurane is the least soluble but it has a pungent odor and animals tend to hold their breath. Death is the result of direct suppression of respiration and of the cerebral cortex, and other vital centers.¹ These agents have been reported to cause no alterations in the histology of most tissues except for congestion of alveolar capillaries.¹³ Both halothane and methoxyflurane have been shown to alter some metabolic parameters^{7,14} and cause lymphocyte proliferation.¹⁵

D. T61

This is an injectable nonbarbiturate agent that is no longer commercially manufactured or available in the United States. It is a combination of tetrazine HCl, butyramide and a curariform drug.^{18,19} According to the European Commission's recommendations for euthanasia, T61 is an acceptable agent if given intravenously and slowly to a sedated animal as it may result in pain if given too quickly to rabbits, rodents, dogs, cats, ferrets and large animals.³ Dogs frequently become distressed and vocalize and move their legs. There is evidence to suggest that respiratory arrest may occur before the loss of consciousness.^{18,19} Hellbreckers et al.²⁰ recently published data from dogs and rabbits disputing this study's findings. T61 is believed to exert both a narcotic and direct effect on the respiratory centers in addition to the paralytic effect of the curariform drug on the respiratory muscles and the heart leading to circulatory collapse, hypoxia and death.^{3,18,19} The side effects of this agent are esthetically unacceptable to many people.

E. KCl OR EXSANGUINATION

Exsanguination is an acceptable method of euthanasia in most species only if done in an unconscious or anesthetized animal.^{1,3} Hypovolemia can cause extreme distress and anxiety and for this reason it can never be used alone. In a research setting this method is a convenient way of doing terminal blood collection on antibody producing animals in order to get as much serum as possible. Bleeding can be done either by venipuncture or cardiac stick. It is not effective in birds because of their tendency for clot formation³ or in reptiles because of their lower metabolic rate and high tolerance of hypoxia. Potassium chloride is a rapidly acting cardiotoxic agent. However, it causes seizures, gasping, and vocalizations and cannot be used as a euthanasia agent in awake animals. It is acceptable for use only in unconscious animals.^{1,3,19}

F. BARBITURATE OVERDOSE

Barbiturates can be used on a wide variety of species and produce a very rapid effect with minimal discomfort to the animal. They result in death by inducing deep anesthesia and unconsciousness with

CNS and respiratory center depression.^{1,4,18,19} Although many barbiturates are acceptable agents, sodium pentobarbital is the most commonly used for euthanasia.⁴ Administration of the drug should be via the intravenous route and never given by intrapulmonary or intracardiac routes in conscious animals because both routes are stressful and painful.⁴ However, the intraperitoneal route is acceptable for rodents, dogs, cats, rabbits and primates. The dosage for rodents is 150-200 mg/kg body weight.⁴ Persons using this technique should be adequately trained in proper restraint of the animal and in proper injection technique to minimize anxiety and stress to the animal. One of the disadvantages of barbiturates is that they are controlled substances and accurate records must be kept of their use. Because they cause relaxation of smooth muscles, splenomegaly is a common gross finding. They are not reported to cause any histologic aberrations to tissues other than vascular congestion of the spleen and lungs.¹³ However, they have been documented to alter endocrine functions as well as metabolic and lymphocytic functions.^{7,14,15}

III. NECROPSY

In order to avoid artifacts caused by autolysis, a necropsy should be done immediately after the animal has died. If this is not possible the carcass should be placed in a leakproof plastic bag and put in the refrigerator until the necropsy is performed. The body should never be frozen if histopathologic examination of tissues is needed as ice crystals form inside the cells and when the body thaws the cells rupture making histologic evaluation difficult or impossible. The bodies of small rodents tend to undergo autolytic changes rapidly.²¹ Within 40–60 min. after death the villi in the small intestine are denuded, the bronchial epithelium separates from the underlying lamina propria, there is cytoplasmic alteration in skeletal muscle fibers and there are changes in the adrenals, lymph nodes and parathyroid glands.²²

There is an old saying that “A necropsy is a message of wisdom from the dead to the living.”²³ The degree of enlightenment derived by this quest for wisdom is dependent largely upon the thoroughness with which the necropsy is performed. A systematic standard operating procedure (SOP) for performing a necropsy must be followed if the results are to be reproducible and uniform.^{21,24} This is a stage at which nontreatment variability can be introduced and interfere with an otherwise reproducible study.²¹ Having a good detailed necropsy form that follows a logical sequence for the recording of gross observations and organ weights is helpful. Accurate morphologic evaluations require that samples be taken from representative regions of each organ.^{21,25} This practice should also be followed when trimming tissues.

Within a research setting, necropsies play a major role in detection and diagnosis of diseases present in laboratory animals. Many diseases can cause significant alterations in the metabolic or immune systems of affected animals and thereby interfere with research studies. Necropsies also play a major role in the quality assurance programs for rodents and other animals in institutions and in screening of vendors. They are also required for toxicologic studies and many research protocols involving biomedical implant devices, surgical models and drug trials.

Necropsies should be performed in a dedicated room with restricted access and used only for this purpose because of the high potential for the spread of pathogenic organisms. Disposable waterproof gowns, latex gloves, plastic shoe protectors and protective eye wear should always be worn as well as masks when indicated. Instruments should be autoclavable or able to be thoroughly disinfected. Containers used for collection of tissues or those used to transport specimens to the lab should be leak proof. Care should also be taken not to contaminate the outside of the containers with blood or tissues. If contamination does occur the outside should be thoroughly cleaned or disinfected prior to removal from the necropsy room. In addition, no one should leave the necropsy room wearing contaminated gowns, boots etc. It is essential that there be adherence to the guidelines from the Centers for Disease Control (CDC) for the handling of potentially harmful pathogenic organisms.²⁶ Biosafety level 2 pathogens are frequently encountered especially with primates and these necropsies should be left to a pathologist or highly trained laboratory personnel.^{26,27} There

should also never be food or drinks in the necropsy room. Once the necropsy is done, the carcass as well as all contaminated disposable items, should be placed in sealed biohazard bags and either incinerated or autoclaved. The necropsy table, walls, counters sink etc. should be thoroughly disinfected. Instruments should be either disinfected or autoclaved.

IV. COMMON SAMPLING PROCEDURES

A. SAMPLING FOR HISTOPATHOLOGICAL EVALUATION

For any morphological, quantitative, or stereological study, one of the most efficient and powerful sampling procedures is the "systematic random sampling" protocol. Within the structure of interest (such as bone, cartilage, ligament or muscle), tissue blocks are sampled systematically and the pattern adopted can be varied among animals. Subdivision into tissue slices and then sample blocks is always carried out at regular intervals according to preplanned systematic protocols.²⁸

Diagnostic necropsies for the detection of diseases are a science and best left to a pathologist who has been trained in the recognition of diseases and altered morphology as well as how to collect the required samples for virology or toxicology testing, etc.^{24,29}

Specimens should be recorded by measuring the size and describing the precise location in a given organ (lung, bone, muscle, or joint), its color, and its consistency. Observations of the gross specimens should be done in purely descriptive terms, not in diagnostic terms.²⁹ The reason for this is that there is significant opportunity for variability in interpretation of many lesions from one observer to another.²⁹ Photography of gross specimens is also a useful way to document findings as well as to obtain teaching materials.

In general, slicing of organs or tissues should be done as sharp dissection with a scalpel blade rather than cutting with scissors that tend to crush the margins of the tissue. Samples for histopathology should be equal to or less than 5 mm thick to permit optimal fixation by the fixative.²² The most commonly used fixative is 10% neutral buffered formaldehyde. Samples for electron microscopy should be 2–3 mm thick. The two most commonly used EM fixatives are gluteraldehyde and Karnovsky's solution both of which are toxic and should only be used in a fume hood.

The methodology for the retrieval and subsequent analysis or testing of orthopaedic implants has been adequately documented.^{30–34} The use of a form on which to record all of the clinical history, radiographic findings and gross observations at the time of removal of the implant and that permits easy conversion of this data for statistical analysis is recommended.³⁰ The gross description should include the appearance of the implant itself, the color, shape, size as well as a description of the fibrous capsule around it and adjacent soft tissues.³¹ In addition, photographing the implant at the time of removal adds a permanent visual record to document these findings. Culture swabs for aerobic and anaerobic (and mycobacteria if indicated) cultures should be taken to document the presence or absence of infection.^{30,35} The implant should be excised along with the adjacent soft tissues and any capsule. A macroscopic examination for cracks, scratches, corrosion etc. may be done using a stereomicroscope.³⁶ If either scanning or transmission electron microscopy are to be performed, it is recommended to do whole body perfusion with buffered gluteraldehyde.^{37,38} Caution should be observed as this is toxic and should be done in a fume hood while wearing gloves and protective eye ware.

For optimal fixation of tissues, especially large sized specimens, perfusion fixation techniques should be used. The procedure should be performed under general anesthesia. For large bones or joints of dogs, goats, or sheep, perfusion fixation should be done through catheterization of major arteries supplying the limb of interest or aorta and vena cava for lower limb fixation.³⁹ The blood is then replaced with heparinized saline, followed by formalin. Lungs should be infused with formalin by injecting it down the trachea and then tying it off.²² Similarly, formalin can be injected into loops of intestine to preserve normal morphology and speed fixation.³⁶ The ideal ratio of the volume of fixative to tissue is 10:1 for formalin and twice that for alcohol solutions.²⁴

B. SAMPLING FOR MECHANICAL TESTING

Specimens of bone, cartilage, tendon and ligament, or other soft tissues to be used for mechanical testing should be harvested with sufficient extra tissues around the area of interest. A hand saw or wire saw is efficient for cutting bone. Soft connective tissue specimens should be placed in saline or PBS or wrapped in saline soaked sponge until testing. Keeping surrounding soft tissue (muscle, fascia, or skin) intact is very helpful for protecting the bone from drying.

C. JOINT FLUID COLLECTION

If it is necessary to collect synovial fluid at the time of necropsy for examining cellular or biochemical components, it should be done first so as not to contaminate the skin with intestinal contents etc. There are a variety of ways to collect synovial fluid depending upon the size and ease of tapping the joint. Sterile techniques always should be followed so as not to contaminate the sample obtained.⁴⁰ The skin should be clipped and a Betadine solution permitted to remain for at least 1–2 min followed by 70% alcohol. Sterile gloves should be used and the area could be draped with sterile gauze sponges or small drapes if needed. Alternately if the joint is not easily tapped or is very small, a sterile surgical approach can be made to the joint and fluid obtained by aspiration after opening the capsule. A sterile 18 gauge needle is preferred. Synovial fluid should be collected into EDTA tubes for submission to the clinical lab for analysis.⁴⁰

Routine examination of synovial fluid from animals should include: a total cell count, a differential count and a ratio of WBC:RBC, mucin clot quality which is a measure of the viscosity and quality of the hyaluronic acid, a total protein and an A/G ratio, specific gravity, color, viscosity and glucose concentration.⁴⁰ Total cell counts from normal joints in animals vary widely from joint to joint and the range for dogs is 0–2900 cells. The microorganisms most frequently recovered from spontaneous septic arthritis in pigs are *Actinobacillus suis*, *Staphylococcus aureus*, *Haemophilus parasuis*, *Mycoplasma hyorhinis* and *hyosynoviae*, *Corynebacterium pyogenes* and *Erysipelothrix rhusiopathiae*. Spontaneous septic arthritis in rats and mice is most frequently caused by *Staphylococcus sp.*, *Mycoplasma arthritidis*, *Corynebacterium kutscheri* and *Streptobacillus moniliformis*.⁴¹ Spontaneous septic arthritis is rare in carnivores except for *Borrelia burgdorferi* in dogs.⁴⁰ In osteoarthritis or rheumatoid arthritis, many biological makers released can be detected by biochemical or immunochemical assays (see Chapters 6, 18, and 19).

D. SAMPLING FOR SEROLOGY

Blood or serum samples are commonly used for routine serological evaluation, bacterial culturing in infections of bone, joint or an implant, or examining markers of pathological conditions (such as serum sulfated glycosaminoglycans released from arthritic joints, acid phosphatase in progressive osteoporosis or bone resorption, or alkaline phosphatase and osteocalcin during fracture healing or bone formation [see Chapters 6, 15]).

At necropsy blood can be drawn from the hearts or from the axillary regions of anesthetized rodents after severing the axillary vessels. Serum samples from animals other than rodents should be drawn before euthanasia is performed. Serum samples should be collected into tubes containing a serum separator. Blood should not be forced through a small gauge needle or it will hemolyze the red cells. The serum should be separated as soon as possible and stored at -70°C .^{42–44}

Serology may be used to diagnose viral infections for viruses that are very difficult to isolate and grow in tissue culture and for viruses with a prolonged course. Multiple samples may be required to demonstrate seroconversion by finding a four-fold increase in viral titer between the acute sample taken during the first two weeks of infection and a convalescent sample taken three weeks later.^{42–44} The use of serology to monitor the health status of rodent colonies is standard operating procedure in many institutions. These samples, typically, are obtained by cardiac stick from an anesthetized rodent as a terminal procedure. Its limitation is that there is a variable lag

time between exposure to the agent and the presence of a detectable titer and, therefore, it is not helpful in animals that become acutely ill and die. Wherever possible, serologic monitoring should be combined with necropsy of sentinel animals and examination for ecto- and endoparasites.

E. SAMPLING FOR CULTIVATION

1. Bacteria

In order to completely diagnose many pathological conditions, it is essential to culture for growth of microorganisms. Obtaining material for bacterial culture must be done in such a manner as to not contaminate the area being sampled which could result in overgrowth of the pathogen by the contaminating bacteria.^{43,44} Ideally, one should try to get needed cultures before the necropsy begins to prevent contamination of the skin lesion or before opening any internal organs. Sterile instruments, gloves and gowns should be used as needed to get the sample.⁴² For most aerobic cultures commercially available sterile polyester swabs such as the Culturette (Becton Dickinson Microbiologic Systems) with transport media are adequate.^{25,42}

Sterile PBS may also be used for short term transport of some organisms but is not acceptable for *Pastuerella*.⁴⁵ If it is suspected that there are only low numbers of organisms present, it is desirable to submit a piece of tissue which can be ground up and cultured.²⁵ Urine can be collected by aspiration from the bladder with a sterile syringe and can be transported to the lab in a sterile screw capped container.^{25,43} Normally sterile fluids like synovial fluid or pericardial fluid can be aspirated into a sterile syringe and then put into screw capped sterile tubes if the volume is small, or if the volume is large, it can be placed into blood culture tubes.^{40,43,44} Blood cultures should be obtained while the animal is alive but if that is not possible, sterile blood can be obtained from the aorta or precava if done under aseptic technique.

Many anaerobic organisms are very fastidious and care should be taken not to expose the samples to oxygen or drying.⁴² Anaerobic samples should be collected into a commercially available system like Anaerobic culturette (Becton Dickinson Microbiologic Systems), the Anerobic pack (Difco Labs.) or the Gas Pack Pouch (BBL Microbiology Systems).^{27,42} Regular aerobic swabs are a poor choice for recovery of anaerobes.²⁷

The use of cotton swabs should be avoided as they contain fatty acids that may retard the growth of bacteria.⁴² Swabs are acceptable for samples from mucosal surfaces in the respiratory, urogenital systems and the conjunctiva.⁴³ Carey Blair transport media should be used for recovery of *Shigella*, *Salmonella*, *Vibrio* and *Campylobacter*.⁴² Abscesses should be sampled by submitting 1–5 ml of pus in a sterile screw capped tube as well as a 1 cm piece of the wall of the abcess as some organisms are not recovered in the pus.⁴²

It is important that bacterial cultures be obtained as soon as possible after death as gut organisms as well as others multiply rapidly after death and invade tissues which may result in overgrowth of the pathogen. In addition, the viability of some pathogens including *Mycoplasma* decreases rapidly after death.²⁴ Sampling techniques should be done so that a sufficient quantity of the material is obtained in order to optimize successful recovery and cultivation of the pathogen and so that the sample collected is representative of the lesion or disease process. Even if excellent collection techniques are utilized, failure to correctly transport or store the specimen prior to submitting it to the lab could result in a negative culture. It is wise to submit the culture as quickly as possible because the viability of some microorganisms decreases rapidly.^{42,43}

2. Fungi

Proper collection of the specimen and how it is transported are of major importance in successful recovery of fungi.⁴³ Aseptic technique should be observed to try to avoid bacterial contamination of the culture. Scrapings from skin or nail lesions should be placed in a sterile petri dish or collected

into mycobiotic or mycosel agar. Pieces of tissue may be submitted in sterile screw capped tubes for mincing or grinding by the lab.⁴³

3. Viruses

The diagnosis of viral infections is dependent upon isolation and identification of the virus in cell cultures or tissues and by detection of an antibody titer in the serum.⁴⁶ The successful recovery and identification of infectious viruses necessitates that the necropsy be done within two hours of death.⁴⁰ In addition, the virus may only be present at very early stages of the disease process. Many viruses are very labile and specimens collected for viral isolation and identification should be kept moist and cold but not frozen.^{44,46} If there is a transit delay time of greater than one hour, samples should be refrigerated at 4°C or placed in a container with cold packs. Samples can be stored at 4°C for up to five days.⁴⁵ Specimens should be collected into special viral transport media that contain either serum albumin or gelatin to protect the virus and antibiotics to stop the overgrowth of the culture by bacteria. Stuart media, Amies media, Liebovitz-Emory media and Hanks balanced salt solution are commercially available viral transport media.⁴² A significant loss of infectious titer occurs when enveloped viruses like *Herpes simplex*, *Varicella zoster* and Influenza are kept at room temperature or frozen at -20°C. This is not true for nonenveloped viruses like adenoviruses or enteroviruses.⁴² Blood for viral isolation should be collected into sterile tubes containing an anticoagulant and refrigerated but not frozen. If it is necessary to store viral cultures longer than five days they should be frozen at -70°C. Coles⁴⁰ has published a list of many of the common animal viruses and what tissues should be submitted to the lab. If the samples must be shipped via the mail to the lab it is prudent to contact the lab at the time of necropsy and follow their directions as to how to package the tissues. Safe handling and processing of samples must be done in full compliance with all applicable CDC regulations.²⁶

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Part II

Evaluation Methods in Orthopaedic Animal Research



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6 Methods of Evaluation in Orthopaedic Animal Research

Yuehuei H. An

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I. INTRODUCTION

Evaluation methods should be carefully selected. Although many sophisticated techniques have been reported, the methods selected for most animal studies should be valid, reliable, efficient, simple, necessary, available, and economical (see Chapter 3). In orthopaedic animal research, clinical observation, radiography, macro-observation at necropsy, histological evaluation, and mechanical testing are used in most cases.

It is impossible to include a detailed description of every method which has been developed in orthopaedic animal research in a single chapter. This review only outlines the more common methods used in orthopaedic animal research and most of them are only briefly mentioned, with the purpose of helping readers find appropriate methods for their projects. One may refer to other chapters for methods of specific interest, for example, the methods for measuring bone apparent density and ash density in Chapter 8.

II. CLINICAL OBSERVATION

Based on the requirements of the study, animals should be weighed periodically. Unless otherwise indicated, animals should be weighed weekly. Any weight loss or decreased weight gain may indicate that something is wrong with the animal. Body temperature should be measured periodically and usually daily for the first week after surgery. The feeding patterns and the amount of daily feces should be also checked. Some experimental studies result in temporary weight loss from stress and other factors.

A normal and healthy animal looks calm, comfortable, has normal reactions to environmental stimuli, and is well groomed. Unlike humans, animals cannot clearly communicate that they are in pain. Therefore, observations should be made with attention to the following signs of pain:¹ (1) activity change (hyperactivity, inactivity, recumbency, withdrawal); (2) vocalization change (increase or decrease); (3) changes of eating and drinking patterns; (4) aggressive or defensive behavior; (5) changes in behavioral patterns (such as grooming, foraging, exercising, or sleep); and (6) change in body temperature. Any animal showing signs of pain should be examined carefully for bone fracture or wound abnormalities, such as infection.

A normal, healthy wound should appear dry with intact sutures. Swelling may be caused by the granulation tissue involved in wound repair (hard swelling) or may be due to hematoma or bacterial infection (soft swelling). Excessive drainage, erythema or soft swelling are indicative of infection (see Chapter 23). Any swelling, especially soft swelling, should be inspected carefully. Joint range of motion should be without limitation. Nonweight-bearing or a carried leg indicates pain or other potential problems such as fracture.

III. RADIOGRAPHY

A. PLAIN RADIOGRAPHY

Radiography is the basic method for evaluating fracture healing and bone defect repair (see Chapter 11 and 13). Pre-operative radiographs confirm normal anatomy and demonstrate the size of the bone, which is very helpful for designing or choosing fixation devices and implants of

appropriate size or shape. In the operating room, roentgenograms using a C-arm unit are useful in determining the quality of internal fixation and the placement of implants. Radiographs should be taken immediately after surgery to examine the position of the fracture or defect and the quality of fixation. Periodic radiographs of the fracture or defect site are essential for monitoring the process of repair (See Chapters 11 and 13 for semi-quantitative radiographic scoring systems for fracture healing and bone defect repair). In addition, angiography has been used to examine the revascularization of a healing fracture or a vascularized bone graft.²

B. HIGH RESOLUTION RADIOGRAPHY AND MICRORADIOGRAPHY

After the animals have been sacrificed, the bone specimens should be radiographed again using a high resolution X ray machine, such as a Faxitron (Hewlett Packard Co., McMinnville, OR). Based on these high resolution radiographs, bone density can be quantitated by measuring the light intensity or illuminance using a digital photometer, such as a Spectron unit (Denver, Co). This technique is commonly called quantitative roentgenographic densitometry. Tiedeman et al.³ found that X ray density correlated well with the ash density and mechanical properties of bone. They believe that this method is superior to radiographic scoring method and is capable of detecting small differences in mineral content even using standard radiographs.

Microradiography (using a high resolution X ray machine) based on thin bone sections (about 0.5–1.0 mm) provides detailed images of bone structures. It can be used in the quantification of bone apposition and ingrowth into an implant.^{4–6} The quality of the bone structure around an implant can also be assessed using this method. A variation called microradiographic videodensitometry is also useful for analyzing bone structures and bone density.^{5,7} The vascularity of repaired bone tissues can be evaluated using microradiography (or microangiography). In this technique radio-opaque substances such as Micropaque, BaSO₄, or lead oxide are injected into the arterial vasculature prior to specimen processing. This allows visualization of the cross-sectioned vessels on thin sections. Vascular trees can also be demonstrated using larger sections.^{8–10}

IV. MACRO OBSERVATION AT NECROPSY

At the necropsy, macromorphology is the essential part of evaluation during surgical or necropsy procedures. The nature, color, and amount of the subjects of interest should be recorded. Regular photography is very important for documentation of what have been found at surgery or necropsy. The specimens containing osseous tissues should be radiographed.

For procedures involving bone, attention should be paid to the size of callus, the alignment of the diaphysis, the positions of the implants or fixation devices, and the surrounding tissues. For conditions involving joints, the morphology of the articular cartilage is the most important consideration. Pathological findings include roughened areas caused by arthritis, cartilage defects, fracture lines, or osteophytes. Also, the size of the joint, joint capsule and synovium; the amount and characteristics of the joint fluid; and the appearance of ligaments, menisci and the soft tissues around the joint should be observed.

Body organs and tissues, such as liver, kidney, lung, or muscle, can be harvested for evaluation of the concentrations of ions released from implanted biomaterials. Using flameless atomic absorption spectroscopy, it has been demonstrated that the lungs of minipigs contain the largest amount of titanium (Ti) ions five months after implantation of Ti screws in the mandible.¹¹

V. STRUCTURAL AND MORPHOLOGICAL EVALUATION

A. MEASUREMENT OF LENGTH AND AREA

Many methods have been used for measuring length and area of bone or the dimensions of other organs or tissues. These methods include direct measurement using a ruler or caliper, measurement

based on radiographs, or the use of specially designed devices. There may be significant inter-method or inter-observer variability, therefore, several observers may be needed to perform the same procedure independently or more than one method may be used. Caution should be taken when inter-study comparison is made, especially if different measurement techniques are employed.

1. Direct Measurement

Most length and area measurements are accomplished reliably by traditional techniques (a ruler or sliding digital caliper) such as the measurements of long bone dimensions (length, internal and external width).^{12,13} A specially designed electronic caliper has also been reported.¹⁴

2. Measurement Based on X Ray Images

Two dimensional measurements are often made from X ray images using a ruler or caliper.¹⁵⁻¹⁸ The percentage of magnification should be considered when using X ray images for measurements. The amount of magnification depends on the distance between the specimen and the film. A metal bar or strip with known length can be used as a reference. It should be placed at the same distance from the film as that of the subject. A standard goniometer is effective for measuring angles based on radiographic images. A custom computer program based on X ray images has been developed in the author's laboratory. It is capable of evaluating the periosteal and endosteal dimensions of the upper humerus and glenoid. Parameters which may be evaluated include humeral canal width, shaft width, tuberosity offset, head offset, radius of curvature of the head and glenoid, head diameter, canal flare index, glenoid height and depth, arc of enclosure, radius of curvature and depth of cancellous bone.¹⁹

The length or perimeter of irregular lines or the area of irregular bone or tissue specimens can be measured using computer image analysis. Most image software has the capacity to measure length and area. Images of interest (photographs, radiographs or prints) can be scanned into the computer, displayed on the screen, outlined and measured. Careful calibration of the software is necessary before accurate measurements can be made.

Measurement of the torsion angle of long bones (in humans) and the femoral angle of inclination (in dogs) has been reported with the use of computed tomography (CT),²⁰ a digital coordinator-goniometer,²¹ and a symmetric axis based method.²²

3. *In vivo* Measurement

In vivo measurement of bone length or limb length is a challenge. Often, soft tissue landmarks are drawn on the skin and the distance between the marks are measured with a tape measure. A technique called kyniklometry has been reported for measuring the distance between soft tissue landmarks on the lower legs of conscious rabbits. It is comparable to X ray stereophotogrammetry.²³ A specially designed goniometer has been reported for the measurement of joint angles in clinical practice.²⁴ Limb circumference measurements are useful for monitoring the progress of a swollen limb, joint, or the growth of a limb. For the quantification of limb circumference, a tape measure is effective. Methods using an electronic digitizer and a mathematical formula for an ellipse (for fetal head and body circumferences) have also been reported.²⁵

B. DISSECTING MICROSCOPY

Dissecting microscopy with magnification up to 5X has been used for examining and photographically documenting the surface morphology of articular cartilage, bone, soft tissues, or implant-tissue interface. Wet specimens can be used, which is an advantage over regular, low magnification SEM. The latter may change the morphology of the specimen surface due to the critical point drying procedure. In the author's laboratory, dissecting microscopy has been found to be useful



FIGURE 1. Implant debris are shown on the bone surface after the implant is removed.

for observing implant debris in the implant bed after the implant is removed (Figure 1). A grading system for evaluation of the severity of arthritis under dissecting microscope was reported by Sommerlath and Gillquist:²⁶ normal cartilage (Grade 0), fibrillation (Grade 1), pannus and fibrillation (Grade 2), superficial clefts (Grade 3), deep localized clefts (Grade 4), large defects (Grade 5), and complete loss of cartilage on the weight bearing area (Grade 6).

C. HISTOLOGY AND HISTOMORPHOMETRY

1. Paraffin Embedding and Decalcification

Paraffin embedding and sectioning remains the most common method for histologic study of soft tissues (subcutaneous tissue, muscle, tendon, ligament), cartilage, and decalcified bone specimens. In the decalcification of bone specimens, the goal is to achieve enough decalcification to allow successful sectioning but to avoid over-decalcification so that cellular details remain intact, thus facilitating successful enzyme and immunohistochemical staining. Common solutions for decalcification include nitric acid, HCl, formic acid, and EDTA. See Chapter 7 and the review by Skinner et al.²⁷ for the details of decalcification methods.

2. Plastic Embedding and Sectioning

Undecalcified preparation and sectioning are specialized procedures for the evaluation of osseous tissues (bone, calcified tissues), dental tissues, and especially specimens containing metal implants (see the review by Sanderson²⁸ and Chapters 7 and 20 for details). In this technique specimens are embedded in plastic media such as methylmethacrylate or Spurr's resin.

There are three major sectioning methods for plastic embedded specimens: (1) direct sectioning using heavy microtomes, (2) "sawing-grinding," and (3) sawing-only. Small undecalcified bone specimens embedded in glycol or methyl methacrylate can be cut using automatic rotary microtomes (such as the Jung Supercut 265) with a tungsten carbide blade (for methyl methacrylate or glycol

methacrylate) or with a large glass blade (for glycol methacrylate). Larger undecalcified specimens can be cut using a sliding microtome (such as the Jung Model K, Heidelberg).

“Sawing-grinding” is the traditional method used with plastic embedded specimens. The specimen is sectioned with a diamond-coated wafering saw (such as the Buehler Isomet 2000, Struers Accutome-5, or Leco VC-50) into 0.2–1.0 mm thick slices. The slices are then glued onto a Plexiglass slide and ground on a grinding machine (such as the Buehler Ecomet 3, Struers Dap-V, or Leco VP-160) to produce 30–100 μm thick sections. In patient and skilled hands, the thickness of the ground sections can be less than 15 μm .²⁹ Well controlled systems with automatic grinding capacity, such as the Exakt sawing-grinding system (Exakt Apparatebau, Germany),³⁰ are also available but are costly. The process is tedious and time consuming. Also, because the slices made before grinding are relatively thick, for small specimens successful cuts have to be guaranteed for production of useful sections for evaluation without wasting.

There are two systems available for sawing-only procedures. One is the modified inner circular “sawing” technique (Fijnmetaal Techniek Amsterdam, The Netherlands) reported by van der Lubbe and Klein (see Chapter 20).^{31,32} This technique can create 12 ± 5 μm thick sections without grinding. Another sawing-only system is a diamond-coated wire saw unit, Histosaw (Delaware Diamond Knives, Wilmington, DE). According to our experience and others,³³ sections as thin as 30 μm can be created using this method in a matter of minutes. The sections are then readily glued onto regular glass microslides, stained, and coverslipped. Because of its simplicity, efficiency, and relatively low cost (15,000 US dollars) it is becoming a popular method for the sectioning of undecalcified or implant-containing specimens. The advantage of these two techniques is that they are capable of sectioning hard tissues or implant-containing specimens without grinding.

Overlapping between the boundaries of implant and tissue may occur due to the porosity of some implant surfaces or because of an imperfect angle between the interface and the sectioning plane (the correct angle is 90 degrees).^{29,34} This phenomenon can be conquered by making the thinnest sections possible and orienting the angle between the interface and sectioning plane at 90 degrees. If a cylindrical implant is used the plane of the cut should be perpendicular to the long axis of the implant. This allows multiple, correctly angled cuts to be made without repositioning the implant. If the cut is made longitudinal to the long axis of the implant, wrong angle phenomenon occur because, theoretically, no right angle sections can be made in this orientation since the thickness of the section cannot be zero (Figure 2). Parr et al.³⁴ demonstrated that interlabel distances were not significantly affected by section thickness. They suggested that the use of microradiographs for histomorphometrical analysis of the implant-bone interface is superior to brightfield analysis because of the low variability of microradiographical data and the added ability to obtain bone mineral density measurements. However, the correct sectioning angle (90°) and the thinnest possible sections always should be obtained in order to utilize the advantages of brightfield observation (visibility of cellular detail and the composition of tissues around the implant).

3. Staining Procedures

Many staining methods are available (see Chapter 7). H&E staining remains the basic and common procedure for most tissues. It can be used for both decalcified and undecalcified specimens. Both Goldner's stain^{30,35} and von Kossa's stain³⁶ allow differentiation of osteoid from mineralized bone matrix. Other common stains for bone sections include Giemsa,^{6,36} toluidine blue,^{29,30,37} methylene blue/basic fuchsin,^{38,39} and Stains-All.^{40,41} Tetracycline labeling is a type of staining which is administered before animals are sacrificed. It is based on the propensity for tetracycline to deposit within bone, and is used for examining bone growth and remodeling. It requires undecalcified sectioning.⁶⁹ Safranin O (for GAG)-fast green,⁴³ Alcian blue (for PGs), and periodic acid-Schiff (for chondroitin sulfate and glycoproteins)⁴⁴ are commonly used in the evaluation of articular cartilage.

Vascular injections of India ink or other dyes prior to tissue fixation are often used for studying the vascularity of various tissues, such as bone or callus,⁴⁵ meniscus, ligament or tendon.^{10,46}

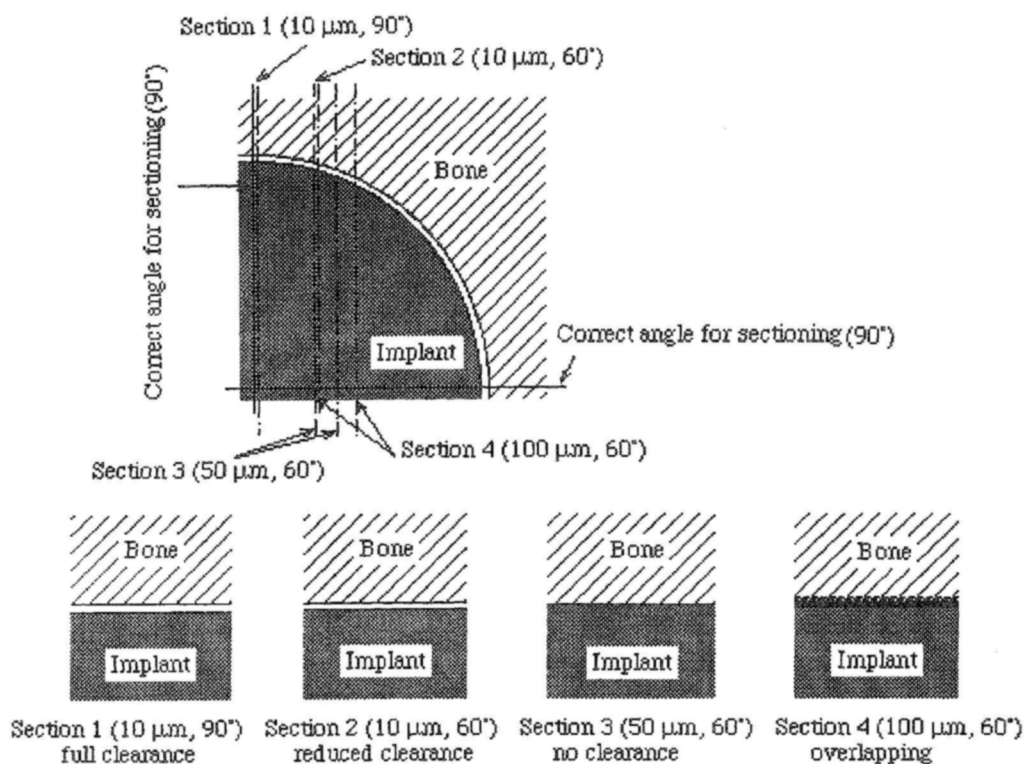


FIGURE 2. Schematic diagram showing the effects of section thickness and the angle of sectioning plane on the appearance of the implant-bone interface (a clearance at the interface is assumed).

Enzyme staining procedures have been developed for localization of alkaline phosphatase (ALP) and acid phosphatase (ACP) in bone, cartilage,⁴⁷ and tissues surrounding an implant.⁴⁸

Immunohistochemical staining (IHCS) techniques have been further developed in recent years. IHCS techniques are used for examining biochemicals in cartilage, bone, ligament, tendon, and other tissues. Immunostaining can be used to identify types I, II and IV collagen, glycoproteins, laminin, tenascin, and fibronectin using plastic embedded bone specimens.⁴⁹ Common macromolecules such as cartilage matrix protein (CMP),⁵⁰ type I, II, and III collagen,⁵¹⁻⁵³ and PGs⁵⁴ have also been successfully localized in cartilage specimens. Other cartilage biochemicals for which IHCS staining methods have been developed include type V, VI, X and XI collagen,^{53,55} chondroitin sulfate, keratan sulfate,⁵⁶ PGs,⁵⁴ stromelysin, tumor necrosis factor- α (TNF- α), TNF receptors,⁵⁷ and fibronectin.⁵⁸ IHCS techniques have been used to demonstrate the distribution of types I, II, and III collagen at the soft tissue-implant interface,⁵⁹ the healing tendon-bone interface,⁶⁰ and the ligament to bone attachment.⁵⁶ IHCS procedures for substance P, tyrosine hydroxylase, and neurofilament have been used to evaluate nerve regeneration.⁶¹ See Chapter 7 for additional staining techniques.

4. Histological Evaluation

Histologic evaluations of bone, cartilage, ligament, tendon, synovium, and other soft tissues have been used extensively in orthopaedic research (see Chapter 7). Observation and characterization are normally done under a light microscope. Descriptive histology and histomorphometry are the two main types of histological study. Depending on the particular situation, either or both may

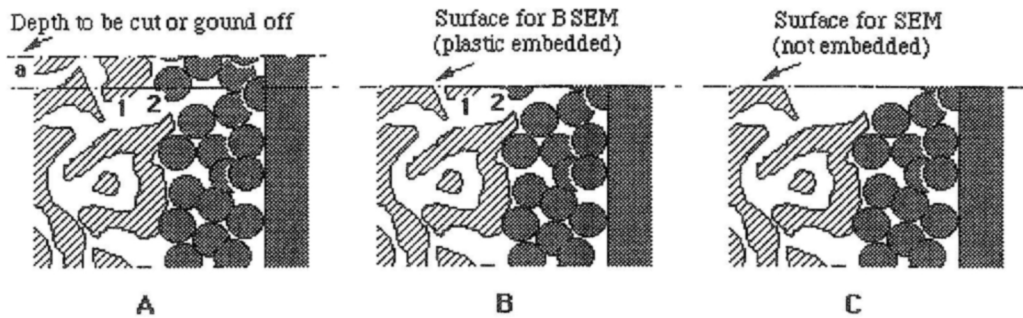


FIGURE 3. Schematic diagram showing the effects of SEM sample preparation on the trabecular bone volume. The upper surface of the tissue block containing implant-bone interface needs to be cut or ground off to create a surface for SEM or BSEM evaluation (A). If the specimen is embedded in plastic media, the end of trabeculae “1” and the lower part of bead “2” will be preserved on the surface (B). If the specimen is not embedded, the end of trabeculae “1” and the lower part of bead “2” may fall off the surface, assuming there are no continuities or connections on the sagittal plane (C).

be used. Descriptive histology is used to give a general picture of the tissue of interest, including the morphology, structure, and arrangement of cells or matrix. Scoring systems are often designed in order to semi-quantify the components of interest. An example of this is the estimation of quantity of new bone formation in a bone defect (Table 3 in Chapter 13). Full bone formation in a defect is scored as 3, moderate bone formation as 2, mild bone formation as 1, and no new bone formation as 0. The data is analyzed using non-parametric analysis of variance. Examples of other such scoring systems can be found in the appropriate chapters. Systems have been developed for evaluation of fracture healing (Table 5 in Chapter 11), bone defect repair (Table 5 in Chapter 13), cartilage defect repair (Table 3 in Chapter 16), and biocompatibility of soft tissue implants (Table 1 in Chapter 20).

5. Histomorphometry

Histomorphometric analysis has been performed using histological sections, microradiographs,³⁴ and backscattered electron microscopic (BSEM) images (on plastic embedded surfaces).^{6,62,63} Standard SEM images of a specimen surface are less favorable for histomorphometric analysis due to the fact that overlying components from adjacent areas are not well demonstrated (Figure 3).

Histomorphometry is a methodology for quantitatively analyzing (1) length (perimeter or boundary), such as the surface perimeter of an implant, (2) distance between points, such as the clearance at implant-tissue interface or distance between the central lines of two trabeculae, (3) area, such as trabecular bone area or repair tissue area, and (4) the number of components of interest, such as trabecular number, vessel number, or cell number.⁶⁴ These parameters are the four types of primary measurements which can be made based on two dimensional (2D) images. Three dimensional (3D) parameters or structures can be calculated or reconstructed from 2D measurements according to carefully considered assumptions. Although accurate 3D data is necessary for proper comparison between different specimens (such as treated and control bone structure), it is often very difficult to reconstruct a 3D structure based on a single 2D image because the structures of most biological tissues (such as bone tissue) are anisotropic. This problem has been partially conquered by the introduction of quantitative CT,⁶⁵⁻⁶⁷ MRI,⁶⁷ and confocal laser scanning microscopy, all of which can easily section and reconstruct the specimen.

In spite of its limitations, 2D histomorphometrical analysis remains a common and useful method for analyzing the structural changes in trabecular bone,^{64,68} the callus composition in healing fracture sites, the repair tissues of bone or cartilage defects, the pathological changes in arthritis, the bone apposition and ingrowth into implant surfaces (see Chapter 21), and the soft tissue-implant interface (see Chapter 20).

Standard nomenclature, symbols, and units for bone histomorphometry can be found in the review by Parfitt et al.⁶⁴ The more commonly used terms for trabecular bone structures include BV (bone volume) or TBA, trabecular bone area, which is the trabecular surface area divided by the total area in mm²; Tb.Th (trabecular thickness, the average thickness of trabeculae in μm); Tb.Sp (trabecular separation, the average distance between trabeculae, representing the amount of marrow space in μm). Common parameters for trabecular bone spatial connectivity include Tb.N (trabecular number, the average number of continuous trabecular elements encountered per unit area), Ho.N (hole number, the average number of holes per unit area), N.Nd (trabecular node number, nodes: trabecular branch points), N.Tm (trabecular terminus number, termini: trabecular end points), and Nd/Tm ratio. Most of the parameters can be measured using specialized imaging software.

Quantifiable parameters can be used for histomorphometrical analysis of fracture callus,⁶⁹ repair tissues of bone defects,^{70,71} cartilage defects,⁷² ectopic bone formation (soft tissue ossicles),^{73,74} mineralized bone, non-mineralized bone, new bone, old bone, chondral tissue, fibrocartilage, hyaline cartilage, and fibrous vascular tissue. Also, parameters for cartilage repair, cartilage thickness and area, degree of attachment, and surface roughness have been developed.⁷⁵

Parameters which are used in the evaluation of experimental arthritis include: articular cartilage thickness and area, synovial cell layer thickness, subchondral bone plate thickness, periarticular bone structure and spatial connectivity.^{68,76–79} Other measurements of synovial or cartilage morphology include synovial cell density, chondrocyte and necrotic cell density, the concentration of lipid-containing cells, and mean surface destruction grade.⁸⁰

In the histomorphometrical analysis of implant-bone interface, the useful parameters are: (1) bone apposition (or ongrowth), which is the fractional linear extent of bone apposed to implant surface divided by the total surface perimeter of the implant (i.e. the surface potentially available for apposition)^{79,81} and (2) bone ingrowth, which represents the amount of ingrown bone per unit of available surface area, porous space and ingrowth depth.^{79,82–84} In the case of bone ingrowth within an osteopenic bone bed, the structure of the bone, represented by TBA, Tb.Th, Tb.N, and Tb.Sp, should be also analyzed.^{79,84} Characterization of the soft tissue-implant interfaces produced in percutaneous and subcutaneous implantation may include quantification of epidermal down-growth, sulcus width, capsule thickness, macrophage density or fibroblast density (see Chapter 20).^{85,86}

In examining the scar tissue within a ligament defect, areas of interest which have been analyzed include blood vessels, fat cells, loosely arranged collagen, disorganized collagen, dense cellular infiltrates, and the mixture of these elements.⁸⁷

For vascular repair, Karim et al.⁸⁸ reported a histomorphometrical analysis of the number of vascular smooth muscle cells, actin stain positive cells, total cells, and the neointimal collagen area.

Like macro-measurements of bone dimensions, there may be significant intermethod or interobserver variability in histomorphometric analysis. When necessary, several observers may need to perform the same procedure independently or more than one method may be employed for comparison.⁸⁹

D. ELECTRON MICROSCOPY

1. Scanning Electron Microscopy

Scanning electron microscopy (SEM) and backscattered electron microscopy (BSEM) are important methods for evaluation of the structure and morphology of bone structures,^{7,68,90} trabecular

bone surfaces,⁹¹ osteoclast-bone interfaces,⁹² cartilaginous surfaces or structures,⁹³ implant surfaces,^{81,94} wear debris,⁹⁵ implant beds,⁹⁶ and implant-tissue interfaces.^{62,97–99} SEM is also a popular method for examining the vascular structure of various corrosion casted tissues (using Mercor injection), including bone,^{46,100} muscle,¹⁰¹ joint,¹⁰² ligament and tendon.¹⁰³ The shortcomings of SEM are that the specimen has to be dried before observation, causing distortion of the original spatial structure and morphology,⁹³ and in some instruments specimen size is limited. The first problem seems to have been solved by the new low-temperature or cryo-SEM system.^{93,104}

2. Transmission Electron Microscopy

Transmission electron microscopy (TEM) is the most powerful method for evaluating the ultrastructure and morphology of large molecules (such as proteoglycans or collagens), subcellular components, cells, and even the implant-tissue interface. Immunolabeled electron microscopy makes it possible to locate biochemicals of interest, such as: PGs within cartilage;¹⁰⁵ collagens within cartilage cells and matrix;^{106,107} and osteopontin, fibronectin, and osteocalcin within the cells or matrix adjacent to implant surfaces.⁹⁹ A relatively new, high voltage electron microscopic tomography method can be used to view the structural relationships between collagen and mineral in bone.¹⁰⁸ The system also has direct 3D imaging capability.

In routine cases, tissues are embedded in Epon or Spurr's medium¹⁰⁹ and ultrathin sections (50 nm to 2.0 μ m) are cut.^{39,99,108,110,111} Section preparation of metal implant-tissue interface had been a challenge for some time. In many cases the implants are removed for easier preparation of ultrathin sections.^{37,112} However, implant removal inevitably damages the implant-tissue interface. Therefore, several methods for preparing tissue ultrathin sections containing the intact implant-tissue interface have been explored. These include: (1) using a soft cored implant coated with a thin layer of metal,^{110,113} (2) removing bulk metal with electrochemical dissolution before embedding,^{112,114} and (3) removing bulk metal by sawing-grinding techniques.^{39,115} Also, 10 μ m sections created by the inner circular "sawing" technique can be used directly for TEM examination.³²

E. CONFOCAL MICROSCOPY

Developed in the 1980s, confocal laser scanning microscopy (CSLM) has already become a new star among the numerous imaging methods used in biomedical research. It has a wide variety of applications and its use has become widespread in orthopaedic research. The technique utilizes a laser beam which can penetrate tissue to a depth of 300–500 μ m and thus reflect images beneath the surface of a specimen. Stored multilayer 2D images can then be reorganized to show 3D or cross-sectional pictures. The advantages of CSLM over the conventional SEM are its ability to view the images within a specimen or cell and the fact that it can be used with wet specimens.

CSLM has been used for viewing the structures at the implant-tissue interface, such as unmineralized bone matrix or mineralized bone.^{116,117} Using CSLM, Piattelli et al.¹¹⁶ found that a layer of unmineralized bone matrix lies at the interface of mineralized bone and titanium screw surface in a rabbit tibial model. Their study revealed that while 40% of the titanium surface contained bone apposition, only 10% of the bone was in direct contact with the screw surface while the other 30% was separated from the surface by an unmineralized tissue layer. Confocal microscopy has also been employed for the examination of cellular survival and proliferation in autogenous flexor tendon grafts in a canine model.¹¹⁸ In another study, CSLM was used to determine the location of viable chondrocytes in frozen and thawed osteochondral articular cartilage.¹¹⁹ An additional use of CSLM is for examining cell location and population in cell-seeded porous constructs.¹²⁰ It has also been utilized to detect the location of type IX collagen in cartilage¹²¹ and type X and XI collagen in the bovine collateral ligament-bone junction.^{122,123}

VI. BIOCHEMICAL METHODS

A. DETECTING MARKERS IN BODY FLUID

Markers are substances which can be detected in various body fluids (including synovial fluid, serum, and urine) and may reflect pathologic conditions. There are three categories of markers which are commonly used in the study of bone and cartilage. These include: (1) metabolic products of cartilage (chondrocalcin [C-propeptide of type II collagen], pyridinium crosslinks, hyaluronic acid [HA]) and sulfated glycosaminoglycans [S-GAG] such as chondroitin sulfate [CS] and keratan sulfate [KS]) and bone metabolic products (osteocalcin [bone gla-protein], hydroxyproline and deoxypyridinium crosslinks); (2) specific enzymes of cartilage (stromelysin or collagenase) and bone (bone-specific alkaline phosphatase, tartrate-resistant acid phosphatase); and (3) hormonal substances, growth factors, or cytokines relevant to cartilage repair (prostaglandin E2 [PGE2], interleukin-1 [IL-1]) and bone repair (endothelial cell-stimulating angiogenesis factor).

Generally, markers of joint pathology (osteoarthritis, rheumatoid or inflammatory arthritis, or joint injury) include elevated levels of S-GAG (CS and KS), HA, chondrocalcin, stromelysin, collagenase, and decreased levels of IL-1 and TNF-OC in synovial fluid, elevated levels of S-GAG (CS and KS), and HA and a decreased level of osteocalcin (due to suppressed bone formation in RA) in serum, and an elevated level of pyridinium crosslinks (due to bone resorption in RA) in urine.¹²⁴⁻¹²⁷ Indicators of bone resorption (in osteoporosis) include elevated levels of tartrate-resistant acid phosphatase and pyridinoline and/or pyridinoline-containing peptides in plasma and elevated levels of fasting calcium and hydroxyproline, deoxypyridinium crosslinks and hydroxylysine glycosides in urine.¹²⁸⁻¹³⁰ Markers for bone formation (in fracture healing, hyperparathyroidism, or hyperthyroidism) include increased levels of osteocalcin, procollagen peptides, and bone-specific alkaline phosphatase in serum.¹²⁸⁻¹³⁰

Enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and high-pressure liquid chromatography (HPLC) are common biochemical methods used for detecting markers in body fluid. Due to their relatively noninvasive nature, these assays are clinically important for monitoring the pathological progress and rehabilitation of certain osseous or cartilaginous conditions. ELISA has been used for evaluating GAG,¹³¹ KS,^{132,133} or CS¹³⁴ in synovial fluid; osteocalcin,^{135,136} GAG,¹³¹ and KS^{135,137} in serum; and telopeptide of type I collagen in urine.¹³⁶ RIA has been used for detecting KS¹³⁸ in synovial fluid; osteocalcin,¹³⁹ carboxyterminal propeptide and telopeptide of type I collagen¹⁴⁰ in serum. Immunoassays are rapid, extremely sensitive and require little specialized equipment. HPLC methods have also been shown to be very effective in quantifying some markers, such as the synovial fluid HA,¹⁴¹ serum PGs,^{141,142} and urinary pyridinium crosslinks of collagen.^{135,143,144}

B. DETECTING BIOCHEMICALS IN TISSUES

Another class of histological stains can be used to localize certain biochemicals in cells or tissues, but they provide only qualitative data. Examples include: safranin O and Alcian blue which specifically stain GAG in cartilage and immunohistochemical stains which can selectively label ALP or ACP in bone and cartilage.

Numerous biochemical tests are available for quantification of biochemical components in bone, cartilage, ligament, tendon, and other tissues. For articular cartilage, DNA content can be evaluated with several different methods,¹⁴⁵⁻¹⁴⁷ the hydroxyproline content (a measure of collagen) with a colorimetric^{148,149} or ³H-hydroxyproline method,¹⁵⁰ the ratios of type I and II collagen with SDS-polyacrylamide gel method,¹⁵¹ PGs or GAG content with a hexosamine method,¹⁵² electrophoresis,¹⁵³ immunoblotting,¹⁵³ or 1,9-dimethylmethylene blue method,^{149,154} PGs synthesis with RIA,^{149,155,156} CMP synthesis with a immunoblotting method,⁵⁰ and fibronectin content with ELISA.⁵⁸ Biochemical methods are also used to evaluate gelatinase activity^{149,157,158} and tissue inhibitor of metalloproteinase (TIMP).^{149,159} Immunochemical methods in cartilage research were summarized by Hardingham.¹⁶⁰

TABLE 1
Mechanical Properties of Tissues and Materials Commonly Encountered in Orthopaedic Research

Material	Ultimate strength (MPa)	Elastic modulus (GPa)	First author, year ^{Ref.}
Biological materials			
Cortical bone	35–283	5–23	An 1998 Chapter 8
Cancellous bone	1.5–38	10–1570 (MPa)	An 1998 Chapter 8
Tendon	10–200	50–1000 (MPa)	Silver 1987 ¹⁷²
Skin	4–14	6–44 (MPa)	Silver 1987 ¹⁷²
Hyaline cartilage	—	0.41–0.89 (MPa, compression)	Mow 1997 ¹⁷⁵
Arterial wall	0.5–1.72	1 (MPa)	Silver 1987 ¹⁷²
Biomaterials			
Al ₂ O ₃	—	550	Wright 1990 ¹⁷⁶
Cobalt alloy	700	200	Wright 1990 ¹⁷⁶
SS	850	180	Wright 1990 ¹⁷⁶
Ti alloy	1250	110	Wright 1990 ¹⁷⁶
HA	600	19	Heimke 1986 ¹⁷⁷
PMMA	35	3	Wright 1990 ¹⁷⁶
UHMWPE	27	1	Wright 1990 ¹⁷⁶
Synthetic rubber	10–12	4 (MPa)	Black 1988 ¹⁷⁴

For bone tissues, assays for DNA, RNA, calcium, ALP, osteocalcin, and collagen content are available.^{161–164} Interestingly, structurally intact and functionally active ALP has even been isolated from clavicle fragments of an Egyptian mummy (before 2000 BC) with an ELISA method using a monoclonal antibody.¹⁶⁵

For ligament and tendon, biochemical methods have been used to evaluate DNA content,^{166,167} collagen synthesis and content,^{166–169} PGs or GAG synthesis and content,^{166–167} collagenase activity,¹⁷⁰ and fibronectin content.¹⁷¹ Determination of the biochemical composition of vasculature also has been reported, including hydroxyproline (for collagen), total tissue protein, DNA and RNA.⁸⁸

VIII. MECHANICAL TESTING

Mechanical properties are the basic parameters of living tissues^{172,173} and biomaterials^{89,174} which are especially important in orthopaedic research. Mechanical properties of bone, cartilage, ligament, tendon, and other tissues have been well documented. Values of strength and elastic modulus of common biological materials and biomaterials are listed in Table 1. Mechanical testing techniques for bone tissues include bending, compression, tension, indentation, torsion, screw pullout test, strain gauges and ultrasonic methods (see Chapter 8). Mechanical testing procedures for bone-implant interface include pushout, pullout, screw pullout test, and a torque removal technique (see Chapter 8). The indentation and confined compression tests are common procedures for testing mechanical properties of articular cartilage (see Chapters 9 and 16). For ligament and tendon, a tensile test should be used (see Chapter 10).

IX. SPECIAL TECHNIQUES

A. AUTORADIOGRAPHY, BONE SCAN, AND SCINTIGRAPHY

Macro- and microautoradiography can be used to examine fracture healing or other repairing tissue in animal models. Briefly, a radioactive chemical (such as ⁹⁹Tcm, ⁴⁵Ca, or ³H) is injected intravenously

before the animal is sacrificed.^{178,179} For macro-autoradiography, bone slices are cut from the fracture site and the slices (or whole bone) are placed on X ray film in a dark room. The film is developed after 12 hrs of exposure. For micro-autoradiography, paraffin sections or plastic embedded sections are made, emulsion coated, exposed for 1 to 27 days (depending on the radiochemical employed) and then developed.^{178,179} The sections are then examined under light microscope. Using micro-autoradiography, DePalma et al.¹⁷⁸ studied the repair process of cartilage defects by quantification of labeled cells in the defects and adjacent marrow areas. Greiff found that radioactivity was localized in the callus and growth plate (the mineralizing regions).¹⁷⁹ Autoradiography has been also used for evaluation of osteoarthritis,¹⁸⁰ inflammatory arthritis (decreased uptake by bone at the early stage of the disease),¹⁸¹ repair tissues in bone defects (using ³H-thymidine),¹⁸² bone formation around implants,¹¹¹ and cellular and collagen distribution within repaired ligaments.¹⁶⁹

Radioactive isotopes such as ⁹⁹Tcm, or ⁶⁷Ca are used for bone scan or scintigraphy. Several hours after injection of the isotope, the radioactivity absorbed by normal bone, necrotic bone, or fracture callus is detected by a scintillation detector. Normally, increased uptake of radioisotope is found at the sites of bone growth or repair with rich blood circulation,^{183,184} and decreased or absent uptake is seen in the areas of ischemic osteonecrosis.¹⁸⁵ This technique has been used for evaluation of bone healing,^{184,186} infected fracture,¹⁸⁶ inflammatory arthritis,¹⁸¹ ischemic osteonecrosis,^{2,185} bone healing around implant,¹⁸⁷ and ischemic muscle lesions.¹⁸⁶

B. COMPUTED TOMOGRAPHY

CT has been used for examining bone structure, bone destruction, new bone formation during fracture healing and bone lengthening in animal models.^{188–190} Noninvasively, CT also has been used to evaluate the revascularization of femoral head necrosis in a canine model (with Micropaque injection).²

Quantitative CT (QCT) is capable of analyzing bone structure, even in small rat bones and is believed to be more sensitive than dual-energy X ray absorptiometry (DEXA).¹⁹¹ The spatial resolution of CT on cancellous specimens can reach 8–80 μ m.^{192,193} The recent development of QCT has resulted in images with high 3D resolution, which may be used for 3D reconstruction of cancellous bone.^{66,67} Based on a 2D array, Feldkamp developed what has become known as the microCT scanner for 3D reconstruction of bone.¹⁹⁴ Another CT method for 3D reconstruction is the X ray tomographic microscope (XTM), which allows *in vivo* evaluation of cancellous bone.⁶⁵

Quantitative CT has also been used for evaluating the density and mechanical properties of bone. It can be applied *in vivo* or on excised bone specimens.¹⁹⁵ CT numbers, image intensity or CT density values (such as Hounsfield units: HU), are measured in the areas of interest. CT density is based on relative attenuation of X rays by a scanned body as compared with attenuation by water. In general, zero HU equals the density of water and –1,000 HU corresponds to the relative density of air. Cortical bone has CT density greater than +1,000 HU and cancellous bone has values ranging from –25 to 714 HU. An average CT value of water is determined for each scan to adjust the systemic error of the machine.¹⁹⁵ By correlation analysis, power functions between CT density and mechanical values (such as strength or elastic modulus), apparent density and ash density of bone can be formulated. Therefore, mechanical values and densities of bone can be predicted by CT values.^{189,195,196} The advantage of QCT is that it can be applied noninvasively and *in vivo*.

C. MAGNETIC RESONANCE IMAGING

MRI provides exceptional soft tissue contrast not afforded by other imaging methods. Therefore, the internal components of synovial joints, i.e., cartilage, menisci, synovium, ligaments, etc. can be seen in exquisite detail.¹⁹⁷ In a canine model, MRI can even distinguish herniated intervertebral disc tissue from scar.¹⁹⁸

MRI has been used for examining changes in bone and cartilage such as the progression of osteoarthritis^{199,200} (see Chapter 18), fracture healing,^{201,202} altered bone structure after bone

biopsy,²⁰³ healing menisci,²⁰⁴ cartilage thickness,^{205,206} and early osteonecrosis in a variety of animal models.^{185,207,208} In a canine model, the necrotic changes of the femoral head are thought to be detectable by MRI within four weeks after the ischemic insult.²⁰⁷ MRI is also useful for detecting early changes of bone marrow produced by bacterial infection.²⁰⁹

Recent studies have shown that MRI also may provide high resolution 3D images of trabecular architecture.^{67,210–212} MRI is believed to be superior to CT and ultrasound methods for this purpose due to its ability to distinguish the boundary between muscle and bone and even between the cortical and cancellous regions within the bone.²¹³ MRI has also been used for evaluating bone mineral density (BMD),^{214–216} and predicting bone elastic modulus (needs further study),²¹⁷ which is very significant for the diagnosis and monitoring of osteoporosis. The latter topic merits increased research attention.

D. SINGLE-PHOTON ABSORPTIOMETRY, SINGLE X RAY ABSORPTIOMETRY, AND DUAL-ENERGY ABSORPTIOMETRY

Single-photon absorptiometry (SPA) and dual energy absorptiometry (DEA) are two noninvasive methods for measuring bone mineral content (BMC) and BMD. They are most commonly applied to the appendicular skeleton.¹⁸⁸ The radioactive sources for SPA are ¹²⁵I and ²⁴¹Am. SPA has commonly been used for measuring the mineral density of the distal radius, ulna, calcaneus and femoral neck. Using formulas generated by regression analysis, SPA can be used to estimate the mechanical properties of healing bone.^{218,219,201} A new method for measurement of bone mass is single X ray absorptiometry (SXA) reported by Borg et al.²²⁰ The SXA device has an X ray tube which emits X rays at an energy level of 40 kVp and 0.2 mA. It has been used to measure the BMC and BMD of forearm bones and the results have shown a very good correlation with the more traditional SPA method.²²⁰

DEA can be performed with either radioisotopes or X rays. When the dual-energy source is derived from X rays, the technique is termed dual-energy X ray absorptiometry (DEXA). A high correlation has been found between DEXA and traditional methods for measuring bone density.²²¹ DEXA has been demonstrated to accurately measure the BMC and BMD of very small areas of interest,²²² such as in rat bone.^{223–225} Like SPA, DEXA has been commonly used to evaluate BMC, BMD, and mechanical properties of normal bone,²²⁶ healing bone^{188,201,227} and osteoporotic bone^{225,228,229} in animal models.

E. ULTRASOUND

Clinically, ultrasound has significant diagnostic values for the evaluation of joint effusions, cartilage defects and joint capsule thickening.²³⁰ Muscle or tendon tears can also be detected and the healing process monitored. Using ultrasound tomography, correct diagnoses can be made for animals with soft tissue abscesses, foreign bodies, hematomas, and soft tissue tumors. The recently developed high frequency ultrasound backscattered microscope has proven to have the ability to visualize the subsurface structures in immature articular cartilage and some of its developmental changes.²³¹ Another recent report has demonstrated the use of echography *in vivo* and *in vitro* for the assessment of changes in articular cartilage and subchondral bone in experimental arthritis models.^{232,233} Ultrasound has also been used for detecting osteomyelitis (OM) in turkeys.²³⁴ OM lesions were recognized as hyperechoic (bright white) disruptions in cortical bone with a specificity rate of 83%.

Quantitative ultrasound (QUS) parameters, such as broadband ultrasound attenuation, ultrasound velocity, and ultrasound attenuation, can be used to investigate bone structure.¹⁹³ In an *in vitro* study on trabecular bone cubes, it was found that ultrasound parameters were significantly associated with bone structural indices, such as Tb.Sp or trabecular connectivity.¹⁹³

QUS is becoming an alternative to photon absorptiometry in assessing bone density. This has been useful in the diagnosis and management of osteoporosis.^{235–237} The diagnostic sensitivity of QUS on BMD is similar to that of DEXA, even on small rat bones.²³⁵ It is also very useful for predicting hip fracture risk.

Ultrasound is a very important tool for measuring mechanical properties of bone as well (see Chapter 8). Ultrasonic techniques offer some advantages over direct mechanical tests for measuring the elastic modulus of bone.²³⁸ Specifically, the specimens can be smaller, with less complicated shapes (cylinder or cube) and several anisotropic properties can be tested using one specimen.²³⁹ Recently, with the combination of vibration analysis and ultrasound velocity measurements, whole bone mechanical characteristics have been assessed *in vivo*.²⁴⁰

F. ARTHROSCOPY

Diagnostic arthroscopy can be used to visualize the surfaces of most components in the joint (articular cartilage, intra-articular ligaments, menisci and synovium).^{241,242} It has also been used for intraarticular surgical procedures, such as ACL transection.²⁴³ The use of arthroscopy is limited to larger animals due to the size of available scopes.

G. MEASURING TISSUE BLOOD FLOW

Several methods have been employed for measuring tissue blood flow, these include: (1) the microsphere method, in which radioactive beads injected into the bloodstream distribute into various organs in proportion to their blood supply;²⁴⁴ (2) the indicator-dilution technique, in which clearance of the indicator is a measure of tissue perfusion;²⁴⁵ and (3) laser-Doppler flowmetry, in which relative perfusion values are determined via detection of blood cell movement. This is done by analyzing the Doppler shift of backscattered light originating from a monochromatic laser light source.^{246,247} The latter is a nondestructive method for determining real-time blood flow in a variety of tissues including muscle and bone.^{247,248} It has been used to investigate the blood flow in bone under different pathological situations, such as osteonecrosis, fracture healing, or arthritis.

X. MOLECULAR BIOLOGICAL TECHNIQUES

Amazing advances in molecular biological technology (MBT) have had an enormous impact on virtually every aspect of medicine, including orthopaedic surgery. MBT is a powerful tool. It has been used for the isolation and analysis of specific regions of chromosomal DNA which indicate various pathologic conditions. In animal models, gene transfer has proven to be an effective therapeutic method for many musculoskeletal diseases. Clinical trials of gene therapy in humans for rheumatoid arthritis are expected to appear soon. Comprehensive reviews of the use of molecular biological techniques in orthopaedic research have been reported by Bridge,²⁴⁹ Sandberg,²⁵⁰ and Shore and Kaplan.^{251,252}

A. BASIC TERMINOLOGY AND METHODS IN MOLECULAR BIOLOGY

The basic terminology given here is adapted from the reviews by Shore and Kaplan.^{251,252} A gene is a unit of heredity, consisting of a segment of chromosomal DNA that is required for production of a functional protein or RNA. The gene contains both coding and regulatory regions. A transgene is a foreign gene which has been spliced into an animal's original genomic DNA. mRNA is a type of RNA that contains protein coding information. Nucleotide sequence refers to the order of nucleotides in a given segment of DNA or RNA. Translocation is the transfer of a portion of DNA from one chromosome to another. A probe is a DNA or RNA molecule that is labeled, or tagged, and can then be used to locate a complementary DNA or RNA strand through

hybridization. Vectors are DNA molecules that are used as carrier molecules for cloned DNA sequences. They contain information which allows recombinant molecules to be replicated in host bacterial cells. A plasmid is a small circular double-stranded DNA molecule which is found in bacteria and replicates independently of the host chromosome. They are commonly used as vectors in molecular cloning. A recombinant DNA molecule is a DNA molecule containing segments of DNA from different origins, such as a piece of human DNA that has been joined to a plasmid DNA. A clone is a term used to describe identical segmental DNA molecules produced by recombinant DNA technique. Molecular cloning is a process by which a specific segment of DNA is isolated and then numerous identical copies, or clones, of that segment of DNA are generated.

Hybridization is the process of matching complementary strands of DNA or RNA or both to form a double stranded molecule. *In situ* hybridization (ISH) is the hybridization of a DNA or RNA probe to a target molecule that has not been extracted from its original cellular location, within a chromosome or in a fixed tissue section.²⁵³ Immunohistochemistry is analogous to ISH for nucleic acids and is used to detect the distribution of a specific protein within a cell or tissue. In immunohistochemistry, a specific antibody serves as the probe to detect the protein of interest. Gel electrophoresis is a method of separating DNA, RNA, or protein molecules based on their size and electrical charge. This technique makes use of the fact that, under an electrical field small molecules migrate through a gel matrix (agarose or acrylamide) faster than larger molecules. Southern blotting or transfer is a technique which is used to transfer DNA that has been electrophoresed through an agarose gel onto a solid support for hybridization. Northern blotting or transfer is the process of transferring RNA onto a solid filter support for hybridization. Western blotting or transfer is the process of transferring proteins that have been electrophoresed through an acrylamide gel onto a solid filter support for detection of a specific protein by antibody labeling.

B. ETIOLOGY AND HISTOPATHOGENESIS

Cloned DNAs can be used to identify the chromosomal location of a gene. This is important in helping to associate a specific gene with a disease. The localization of specific genes has been accomplished by determination of critical, consistent karyotypic breakpoints which are characteristic of specific histological types of mesenchymal neoplasms.²⁴⁹

Several methods are available for localization of specific genes, translocated chromosomes, or chromosomal breakpoints, for example: fluorescent *in situ* hybridization (FISH), in which a fluorescently labeled probe is allowed to hybridize to specific chromosomes and thus determines their gene location microscopically.²⁵² Also, *in situ* hybridization (ISH) is capable of detecting mRNA for a specific gene, using nucleic acid probes which are complementary to and hybridize with the mRNA. Because it is performed on tissue sections, ISH localizes the mRNA to individual cells within the tissue and thereby allows investigation of the spatial distribution and heterogeneity of expression of particular genes within a population of cells. The method described by Hicks et al.²⁵³ is a typical example of ISH. It describes, in detail, a method for performing ISH on skeletal tissue cells (growth plate cells) using synthetic oligonucleotide (a short sequence of nucleotides) probes. This technique is currently being translated into diagnostic practice.²⁵⁴

Specific chromosomal abnormalities have been associated with a number of skeletal neoplasms. In Ewing's sarcoma or Askin tumor, a rearrangement of chromosome 11 and 12 t(11;12) has been found. A translocation of chromosomes X and 18 t(X;18) is characteristic of synovial sarcoma of the extremities. In osteosarcoma, chromosomal regions 1p11-13, 1q10-12, 1q21-22, 11p15, 12p13, 17p12-13, 19q13, and 22q11-13 are frequently rearranged.²⁵⁵ Also, overexpression of the cyclin G1 gene is frequently observed in human osteosarcoma cells.²⁵⁶ In 50% of solitary lipomas, a structural rearrangement of 12q13-14 has been detected. Many chromosomal translocations are tumor-specific.²⁴⁹ The identification of clonal chromosome abnormalities has also been reported in association with orthopaedic conditions, such as proliferative fasciitis, Dupuytren contractures and

osteochondromatosis. This technique has provided new evidence for a neoplastic origin of these lesions in contrast to previous theories of reactive, developmental, or hormonal etiologies.

Collagens is the basic component found in the extracellular matrix of healthy tissues. Mutations in collagen structures cause a variety of diseases that include osteogenesis imperfecta (mutations in one of the two structural genes for Type I procollagen), chondrodysplasia (mutations in genes for Type II collagen), and possibly, some forms of osteoporosis, osteoarthritis, and aortic aneurysms (defects in Type I, II, or III collagen, respectively).^{257,258} Many disease phenotypes have been produced in transgenic mice by introducing mutated collagen genes. Such animal models have proven to be excellent tools for investigating the consequences of mutations in collagen genes and have helped to identify additional diseases caused by collagen defects.²⁵⁹

Gene expression during fracture repair has been reviewed by Sandberg et al.²⁵⁰ They summarized the regulation of genes coding for extracellular matrix components and growth regulatory molecules during fracture healing. The information available focuses on the sequential expression of genes coding for collagens, PGs, and other matrix proteins during secondary (callus) healing. The temporal and spatial distribution of different connective tissue components (mesenchyme, cartilage, and bone) is closely linked to the expression of genes coding for their characteristic constituents. The current and the near future development of MBT will provide answers to some persistent questions in fracture healing.

C. DIAGNOSIS AND PROGNOSIS

The examination of specific genes, chromosomal translocations, and gene expression allows direct and accurate diagnosis of many clinical conditions (such as cancer). It also permits characterization of the changes which occur in different stages of tissue repair (cartilage or bone repair). Some especially powerful applications include: diagnosing specific disease processes which may have varying histological characteristics; distinguishing certain neoplasms which have similar histological appearances; and distinguishing certain benign tumors from their malignant counterparts. Chromosome analysis and reverse transcription-polymerase chain reaction (PCR) methods are commonly used for disease diagnosis and follow-up.

Cytogenetic findings obtained after clinical diagnosis have many applications. They can be used to: (1) serve as indicators of disease progress (such as the transformation of a benign tumor into its malignant counterpart or transformation from one tumor into another); (2) indicate the need for a change in treatment plan (as would be the case for a malignant transformed tumor); and (3) monitor the effectiveness of treatment (by detecting reduction or elimination of a specific tumor or monitoring the changes seen in different stages of fracture healing).

D. GENE THERAPY

Gene transfer is a procedure in which certain therapeutic genes are administered locally or systemically. This can be accomplished by direct introduction into diseased sites (such as tumor, bone or cartilage defects, or arthritic joints) or by *ex vivo* transfer into cells or tissues which are then transplanted into the diseased location. Systemic administration allows the secreted gene products to enter the circulation and thereby reach the disease sites. A variety of vectors, including retroviruses, adenoviruses, herpes simplex viruses, and liposomes, as well as naked DNA, have been used to deliver genes into living subjects.

Several gene transfer methods aimed at the treatment of osteosarcoma have been reported. Ko et al.²⁶⁰ reported that the recombinant adenovirus (Ad) vector containing the thymidine kinase (TK) gene driven by the osteocalcin (OC) promoter (Ad-OC-TK), when delivered concurrently with acyclovir, is highly selective in blocking the growth of osteosarcoma in a nude mouse model. Another recent report showed the efficacy of a high-titer antisense cyclin G1 retroviral vector in a nude mouse model of osteosarcoma.²⁵⁶

A number of highly specific post-translational enzymes involved in collagen biosynthesis have recently been cloned. There has been increasing interest in the possibility that the unique post-translational enzymes involved in collagen biosynthesis may offer attractive targets for specifically inhibiting excessive fibrotic reactions in a number of diseases. Several investigations also have suggested that it may be possible to inhibit collagen synthesis with oligo-nucleotides or antisense genes.²⁵⁸

Gene therapy offers novel possibilities for the treatment of inflammatory or rheumatoid arthritis.²⁶¹ Presently, *in vitro* and *in vivo* investigations are directed toward gene transfer in order to allow the delivery of genes whose products possess antiarthritic properties, such as IL-1 receptor antagonist gene or tumor necrosis factor inhibitor (TNFI) gene.²⁶² Gene transfer using a retrovirus vector has been successful in achieving high intraarticular transgenic expression of an IL-1 receptor antagonist. This has had promising antiarthritic effects in animal models. Soon a human trial using this principle is expected to appear.²⁶¹ A similar study in dogs has demonstrated that the injection of transduced synovial cells (with human IL-1a gene) can slow the progression of experimentally induced osteoarthritis.²⁶³

By gene transfer, it is also anticipated that therapeutic growth factors to be expressed *in vivo* at high concentrations for an extended period of time in order to enhance tissue repair. The feasibility of this concept has been tested by Kang et al.²⁶⁴ in an articular cartilage defect model. They found that rabbit chondrocytes were susceptible to *in vitro* retrovirally mediated gene transfer, and that transgene expression persisted for four weeks following allotransplantation into full-thickness articular defects. This is a new, potentially very effective, approach to orthopaedic tissue repair. Further investigation is needed.

In the area of tendon and ligament repair, a number of growth factors have the potential to enhance the healing process, but they are extremely difficult to deliver clinically. Several studies have demonstrated that a possible solution to this problem is the use of gene transfer. Direct injection of an adenoviral vector carrying the lacZ gene and allotransplantation of lacZ containing tendon fibroblasts has resulted in lacZ gene expression throughout the body of the tendon itself. The effects lasted for six weeks, which may be long enough for clinical usefulness.^{265,266}

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7 Histological Study in Orthopaedic Animal Research

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I. INTRODUCTION

The focus of this chapter is to provide a practical selection of useful guidelines and technical procedures relevant to the histologic study of bone, cartilage and associated soft tissues in the orthopaedic research laboratory setting. Occasional key references are cited, but the limited scope of a single chapter does not permit inclusion of all the publications which have provided technological advances and useful methodologies in bone and cartilage histology throughout the years. Important

reference sources for detailed discussions of bone histology and detailed methods for histologic procedures are found in a number of excellent publications which are also highly recommended to the interested reader and researcher.¹⁻⁵

Histology continues to remain a valuable tool in orthopaedic research. Specialized histologic techniques for bone and cartilage have historically built upon the methods developed for examination of bones from clinical conditions such as bone tumors, skeletal dysplasias, and, since the mid 1960s evaluation of undecalcified bone biopsies, from patients with osteoporosis, osteomalacia, or other metabolic bone diseases. However, routine clinical laboratory histology methods used in the pathology laboratory are sometimes inappropriate for the specific needs of orthopaedic examination of experimental bone and cartilage specimens. In the sections below, guidelines have been developed to assist the researcher in choosing decalcified or undecalcified bone processing schemes, and to suggest protocols best suited for the needs of the investigator.

Interest in the histologic examination of bone continues to grow as evidenced by a recent issue of the *Journal of Histotechnology* totally devoted to topics of bone and cartilage studies.⁶ Such interest bodes well for the continued development and utilization of specialized techniques for study of these challenging tissues. The authors wish to thank all the investigators who have contributed valuable technical and interpretative methods for the study of bone, cartilage and associated soft tissues; not all important work was able to be cited in the space of this chapter. We hope that the reader will find the focus of this chapter of practical value in the study of bone, cartilage and associated soft tissues.

II. EXPERIMENTAL DESIGN

The focus of this chapter is to provide selected helpful guidelines of practical use to the orthopaedic researcher with little or no previous histologic experience, and to provide specialized information for the investigator with more advanced needs. The importance of early planning to define the histologic endpoints of interest and choice of the histologic methods which will achieve the desired endpoints is emphasized.

A. DESIRED ENDPOINTS FOR EXAMINATION

It is very important to have a specific histologic region of interest defined at the start of the study. The first step is to clearly define a sampling site. For orthopaedic studies, the sites of interest can range from cartilage (e.g., changes in the physis or growth plate during surgical procedures, administration of drugs or metabolic agents, or unweighting of a limb; changes in articular cartilage with aging or disease), to bone (trabecular [metaphyseal or epiphyseal], cortical [including the endosteal or periosteal envelopes]), or soft tissues surrounding the bone and cartilage. For each of these sampling sites, a well defined experimental plan is needed to ensure that the correctly harvested and trimmed specimen is submitted for the appropriate histology method.

Also critical is appropriate careful dissection of the site of interest at harvest prior to submission to histology. If a study requires the examination of multiple sites and the exact anatomic orientation and location of specific regions are critical, histologic marking dyes are very useful aids; these come in several colors and will not harm the histologic preparation, and are best applied during specimen harvest by the investigator (see Appendix 1).

Pilot studies during which the exact skeletal site of interest is harvested using the exact conditions which mimic the planned experiment are possibly the most often overlooked part of the early experimental plan. The ability of a histology lab to successfully deal with bone and cartilage specimens varies with the research setting and the technical expertise available. If the orthopaedic department has access to a lab experienced in bone and cartilage histology, the investigator's planning task is much easier. If, on the other hand, specimens are going to be submitted to a general histology laboratory, early planning and practice specimens are well worth the extra effort.

TABLE 1
Key Questions in the Early Experimental Design Involving the Histologic Study of Bone and Cartilage

Histologic Feature of Interest	If “Yes,” then	If “No,” then
Are you interested in osteoid seam width or area?	Utilize undecalcified processing and embedding in methyl methacrylate.	Decalcify and embed in paraffin or glycol methacrylate.
Are you interested in measurement of bone formation or mineralization?	Double labelling with tetracyclines prior to animal euthanasia and then use undecalcified processing and embedding in methacrylate. Record dates of label administration and date of euthanasia.	Decalcify and embed in paraffin or glycol methacrylate.
Are you interested in histologic studies of surgical sites?	Consider pre-op and post-op radiographs and photographs of site of interest.	
Be sure that histologic embedment uses appropriate orientation to locate sites of interest (use marking dyes).		
If you are decalcifying your specimens, will you need to localize acid or alkaline phosphatase?	Use a decalcification method which does not block histochemical localizations.	
If you are interested in cortical bone, are you interested in osteoid seam width or area, bone formation or bone mineralization? Cortical endosteum vs. periosteum?	Use methyl methacrylate preps of longitudinal sections of the bone for tetracycline labelling; for cortical cross-sections, use hand ground thin sections (without decalcification).	Decalcified paraffin-embedded cross-sections and longitudinal sections can be used.
Are you interested in the periosteum of the cortex?	Be sure that during harvest of the specimen the periosteum and a thin layer of adjacent muscle is left intact.	

It is also worth noting that confirmation of normal anatomy prior to surgical procedures is important if costly animal models and/or costly research studies are involved.

B. DECALCIFY OR NOT DECALCIFY

Table 1 summarizes frequently encountered questions which the researcher must answer before a new project is started. There are several reasons for choosing methods which do not decalcify specimens prior to embedment: (1) to differentiate osteoid seams from mature mineralized bone; (2) to qualitatively or quantitatively assess tetracycline incorporation to study the rates of bone apposition, mineralization and formation; (3) to localize certain enzymes such as alkaline and acid phosphatase or other substances which can be histochemically localized in methacrylate embedded specimens; and (4) to analyze implant-containing specimens. The latter include implant-bone specimens and implant-soft tissue specimens. When a study does not focus on tetracycline labelling of bone, does not require for localization of ezymes, or the specimen does not contain a metal implant, decalcification of bone specimens may be the method of choice.

C. BONE LABELLING

Bone is unique as a tissue in that we can use time-spaced tetracycline and other labels to determine dynamic indices of bone formation and mineralization (Figure 1). It is interesting to note that Klein et al have modified the tetracycline bone labelling scheme and developed a method to assay bone resorption *in vivo* with ³H-tetracycline.⁷

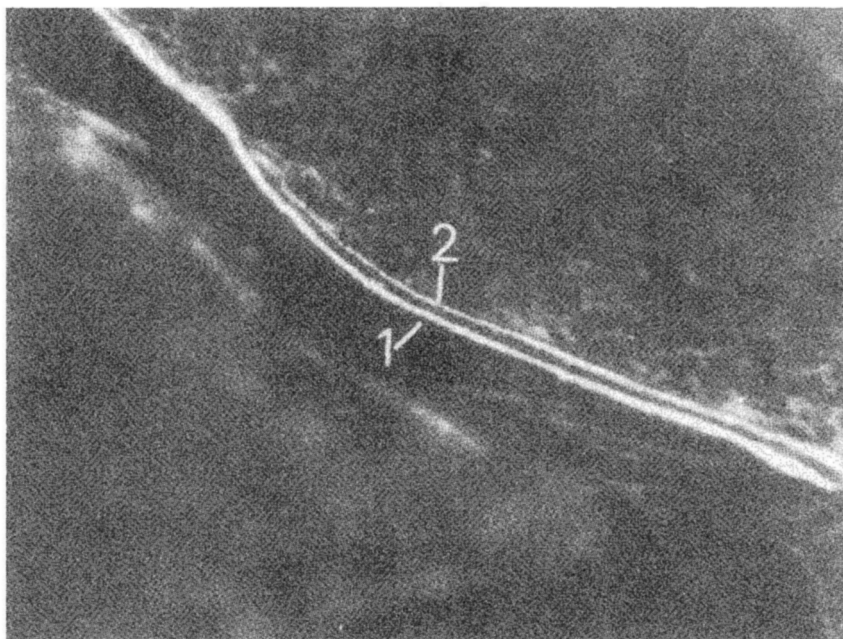


FIGURE 1. Photomicrograph of a methacrylate-embedded biopsy of the anterior iliac crest of a 56-year-old female who received two courses of tetracycline prior to biopsy. When viewed with fluorescence microscopy, double labels of tetracycline mark the bone sites where active bone formation and mineralization were present when the first (1) and second (2) courses of tetracycline were administered. (Unstained section under fluorescence, $\times 140$.) (From Gruber, H. E., et al., *Semin. Haematol.*, 18, 258, 1981. With permission.)

Many bone labelling agents are available.⁸ The commonly used tetracyclines for humans (Achromycin, Declomycin, oxytetracycline) can readily be used for bone labelling in animal models. Other agents, such as alizarin red S, can also be used.⁸⁻¹⁰ Most tetracycline antibiotics form stable tetracycline-calcium chelates which fluoresce intensely at wavelengths readily obtained with the standard fluorescent microscope. These chelates form only at sites of new bone deposition where the bone contains 20% or less of the maximum mineral content.¹¹ These chelates are locked into bone during further mineralization and remain in the bone until it is later resorbed by osteoclasts. Tetracyclines also mark the mineralization front in calcifying cartilage. By use of two different tetracyclines which fluoresce different colors, first and final labels can be distinguished in bone forming sites which show only one label incorporation. Such sites either stopped forming after incorporating the first label, or started forming just before administration of the final label.

Usually two injections are used, time-spaced to ensure that the labels are adequately separated for clear microscopic visualization. If your experiment uses an unusual animal model, or if you are studying very old animals with low bone turnover, a pilot study should be carried out testing the labelling intervals for the labelling agents. Labelling studies require that the dates of administration of the labels and the date of euthanasia are recorded. Critical is the processing of specimens in an undecalcified manner with embedment in methacrylate (see below).

III. SPECIMEN HARVESTING

Bone and cartilage are among the most challenging of tissues for histologic examination. Access to the appropriate tools and equipment is a critical aspect of successful orthopaedic histology. As with all tissue obtained for microscopic study, it is important to obtain the specimen quickly and place it in the appropriate fixative as soon as possible. During harvest of large bone specimens

which require harvest with an autopsy saw or other saw, it is important to keep the specimen moist during harvest and sawing. Subdivision with small bench saw or other saw unit is sometimes necessary. If possible, this should be done under a fume hood with the specimen kept wet with fixative during the separation process; bone fragments caused by cutting should be gently removed. If ventilation is not available, buffers or saline can be used to wet the cut surface of the bone in place of formalin.

For studies of iliac crest bone and other trabecular bone sites which crush easily, the researcher should consider the advantages of using an electric drill for transiliac and vertical bone core specimens. If manual methods are to be used, Jamshidi core needle biopsies can be obtained. In both instances, it is important to develop good skill in minimizing drilling to prevent heat and crush artifacts in the specimens. Cores must also be of adequate size to permit reproducible histomorphometric measurements if quantitative analysis is needed.¹

Once the gross specimen has been obtained, smaller saws and tools in the histology lab can be used to further trim the specimen. As mentioned above, colored dyes can be a useful aid for maintaining bone orientation and surgical landmarks. Cork sheets placed under specimens during sawing help to absorb vibration and make the specimens easier to handle. Small hand held saws and even dental drills can be adapted with diamond-coated circular saw blades for fine trimming of specimens. Such trimming should be done under a fume hood with care taken for safe operation of drills and protection against bone fragments dislodged during sawing. A diamond-coated wire saw (Histosaw, Delaware Diamond Knives, Wilmington, DE) is very useful for precise trimming of bones prior to and after methacrylate embedment.

IV. FIXATION AND TRANSPORTATION

Older studies utilized ethanol as a primary fixative because of concerns that aqueous based formalin fixatives might produce some leaching of tetracycline labels from the mineralizing front of bone specimens. Although still used by some investigators,¹ ethanol does not give good cellular preservation. Currently in most laboratories 10% buffered formalin is used as a general primary fixative. Studies using *in situ* hybridization may require other types of fixatives.¹² Such studies again require careful pilot trials to ensure appropriate processing procedures.

As mentioned above, it is important with large specimens to ensure appropriate penetration of the fixative solution. Vacuum should be used at the start of fixation. A vacuum pump attached to a desiccator with inlet and outlet ports provides a simple way to pull a vacuum on bone specimens to ensure adequate penetration of fixative into the tissue. Vacuums should be pulled until bubbles cease to emerge from the specimen. For large specimens, it may be advantageous to let specimens remain in fixative under vacuum for the whole period of fixation. For large specimens, trimming the muscle carefully from the bone aids in penetration. If only the diaphyseal bone is the site of interest, it is helpful to remove the proximal and distal metaphyses, which provides openings for access of fixative to the interior of the diaphysis. A general rule is that the volume of fixative should be at least 10 times that of the volume of the specimen to be fixed.

Fixation time is a critical issue for bone and cartilage as it is for histological procedures with other tissues. The two enzymes of major interest in bone histology, acid phosphatase and alkaline phosphatase, show different sensitivities to exposure to formalin.¹³ Therefore, a total fixation time of 4–6 hours is recommended. If transportation is needed the specimen should be shipped in 70% ethanol. Specimens can be stored in 70% ethanol (higher concentrations tend to cause the specimen to become brittle).

V. SPECIMEN PROCESSING AND SECTIONING

A. DECALCIFIED SPECIMENS

Decalcification procedures in general use either acids which react with the calcium in bone to form soluble calcium salts, or chelating agents which complex the calcium ions. Acid decalcifying agents

are known to have the drawback of altering the staining properties of the embedded bone.¹⁴ Some decalcifying solutions also contain formalin, and thus increase the possibility of aldehyde groups increasing in the tissue and blocking reactions.

A large number of commercially available decalcification solutions offer a wide range of decalcification times. It is important to remember that many commercial products have been developed to be used for rapid decalcification of large specimens in the clinical pathology lab; these products should not automatically be assumed to be suitable for specialized orthopaedic research applications. In our laboratory, we routinely use a formic acid decalcification method (Appendix 1) which preserves the ability to localized tartrate-resistant acid phosphatase and alkaline phosphatase, and can be used for anti-Factor VIII immunohistochemical localization of vasculature.

If the experiment involves specialized histology, such as localization of tartrate-resistant acid phosphatase or alkaline phosphatase, of *in situ* hybridization studies, it is important that the decalcification method is compatible with the special techniques. Although rapid decalcification solutions are quick, there is the danger that they have destroyed the ability to localize key enzymes and to do other special procedures on the decalcified tissue. An alternative to paraffin embedding of decalcified tissue is embedment in glycol methacrylate; this affords a crisper resolution of cellular detail (see below) and is often overlooked as a method of choice. It is again advisable that a pilot study be used to ensure compatibility of the chosen decalcification/embedment method with the desired histologic outcome.

Although raising the temperature can hasten decalcification, it also may further deteriorate cell and tissue details. Placing the specimen in the decalcifying solution on a shaker and frequently changing the solution are important. Generally a 20:1 decalcifying solution:specimen volume ratio is recommended.¹⁴

The two most frustrating and disastrous outcomes from improper decalcification are (1) incompletely decalcified specimens which are embedded too soon and are impossible to section successfully, and (2) specimens which have been decalcified in a harsh manner so that cellular detail is lost and enzyme/immunohistochemical localizations cannot be performed.

Several methods are useful to determine whether a specimen has been completely decalcified and is ready for paraffin processing. For long bones of small mammals, a test can be made by testing the cutting of the cortex. If a scalpel cuts it easily, decalcification is complete. For larger bones, if one has access to an X ray unit, taking a radiograph of the specimen is a useful technique. The bone should have the same radiological contrast as surrounding muscle in the appropriately decalcified specimen. Chemical tests to determine if calcium is still being removed from the specimen (indicating incomplete decalcification) include the calcium oxalate test,¹⁴ and determination of calcium content of the solution with atomic absorption spectrophotometry.¹⁵

For decalcified specimens, regular microtomes are efficient to cut 3–6 μm thick sections.

B. UNDECALCIFIED SPECIMENS

1. Embedding of Undecalcified Specimens

In the past, various plastic embedding techniques have been successfully applied to bone and teeth.¹³ Older methodologies relied upon UV polymerization with the specimens cooled in a water bath during polymerization. More recently, methods to remove commercially added inhibitors, and use of chemical initiators and catalysts, have greatly simplified plastic embedding. With careful variations on protocols, even large specimens, such as intact canine spine, can be embedded.¹⁷

Processing of undecalcified bone specimens is still a relatively long process. In Appendix I methods are summarized for our procedures for glycol and for methyl methacrylate embedding. Glycol methacrylate embedding results in good cartilage preservation and good preservation of osteoblast-associated alkaline phosphatase. Methyl methacrylate embedding results in a harder plastic providing superior support for cortical and trabecular bone. The goal is to match hardness

of the embedding support medium to the hardness of the bone or cartilage in order to produce successful sections. The reader is also referred to previously cited sources for other useful embedding methodologies, such as Spurr's embedding method.

Large bone specimens are technically challenging both for decalcified and undecalcified preparations. The three most important steps are to ensure adequate fixation of the specimen (without masking desired enzymes or antibodies by exposure to aldehydes), and, for decalcified preparations, to achieve decalcification without loss of special staining/enzyme capabilities, and to obtain adequate infiltration of the specimen for either paraffin or methacrylate processing. As previously noted, a pilot study where the actual skeletal site of interest is used for laboratory practice is strongly recommended.

Our laboratory has recently reviewed large specimen methods in our study of the canine spine and has developed a procedure for infiltration and embedment of large bone specimens.¹⁷ Pulling a vacuum on large specimens is very important to aid in infiltration of solutions and of the final polymerization solutions. If one is solely interested in the epiphysis and metaphysis, the diaphysis can be cut at mid-shaft; this provides an open face of bone and marrow into which solutions can more easily penetrate. Infiltration under vacuum can also be used. The presence of bubbles in the final polymerized plastic block indicates a rapid, uncontrolled polymerization which generated heat and damaged tissue. For methacrylate embedments, a heat sink can be incorporated into the procedures. Water is an excellent heat sink provided the surface is sealed with oil so that humidity does not build up in the polymerization chamber. Large specimens also sometimes require special methods to mount them for microtome sectioning; listed in Appendix 2 are useful molds and sealants which allow firm specimen anchorage during sectioning by large microtomes. Care should always be taken in trimming the initial submitted specimen if the cortical periosteum is of interest. Muscle should never be pulled from the cortical surface; gentle trimming of muscle layers with a scalpel ensures that a thin layer of muscle can be left overlying the periosteum.

2. Sectioning of Plastic Embedded Specimens

Based on the specific purpose of the study, the size of the specimen, or the existence of an implant, the following sectioning methods may be used for plastic embedded specimens.

Small undecalcified bone specimens embedded in glycol or methyl methacrylate can be cut using an appropriate motor-driven microtome and tungsten carbide knives (for methyl methacrylate or glycol methacrylate) or large glass knives (for glycol methacrylate).

For ground sectioning, traditionally, a slow speed diamond saw is used to cut approximately 1.0 mm thick sections off non-embedded or plastic embedded specimens. The sections are then glued onto plexiglass slides for grinding by hand on a grinding wheel to produce sections with average thicknesses of 25–100 μm . Subsequently, the sections are stained with a variety of procedures for microscopic evaluation or viewed directly with UV microscopy for identification of fluorescent labels.¹⁸ In most places, this method remains the routine selection for study of plastic embedded specimens. It does not, however, provide good resolution of histologic detail at the cellular level because of the thickness of the specimen.

A diamond-coated wire saw, Histosaw (Delaware Diamond Knives, Wilmington, DE), is a relative new sectioning system. According to our experience and others', sections with a thickness down to 30 μm can be created using this method in a matter of minutes. The sections are then readily glued onto regular glass microslides, stained, and coverslipped.

C. SOFT TISSUES

Muscle, tendons and ligaments can usually be processed as other soft tissues either in paraffin or in glycol methacrylate. Orientation is critical for muscle, and in studies either longitudinal or tangential sections of muscle bundles may be desired. Tendons and ligaments can curl and twist

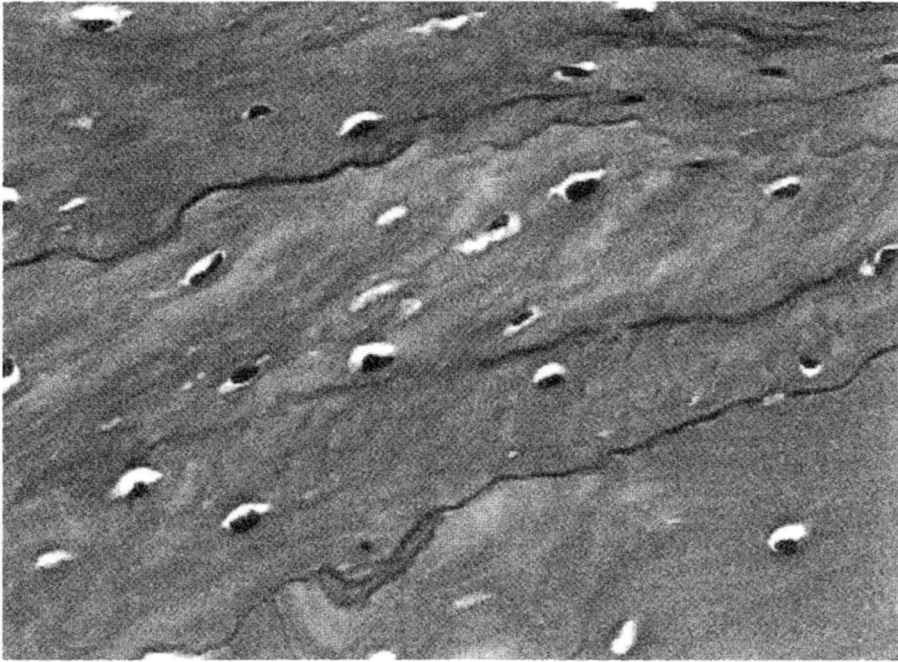


FIGURE 2. Photomicrograph illustrating good visualization of cement lines in a decalcified specimen of rat femur stained with hematoxylin and eosin ($\times 420$).

during processing; this can sometimes successfully be dealt with by anchoring the ends to a wooden applicator stick or tongue depressor to maintain specimen orientation during processing and sectioning.

If the insertion sites onto bone are the regions of interest, specimens must either be decalcified or processed through plastic undecalcified. Synthetic materials utilized to reconstruct tendons or ligaments may require longer infiltration processing intervals for paraffin or may alternatively be processed in methacrylates.

VI. STAINING TECHNIQUES

A. STAINING OF BONE AND CARTILAGE

Many excellent reviews have previously presented selected methods for staining bone and cartilage.^{4,14,19}

1. Decalcified Sections

Many of the routine trichrome, hematoxylin/eosin, toluidine blue and other stains have been adapted for study of bone and cartilage in paraffin sections (Figure 2). Methylene blue/basic fuchsin and other metachromatic stains are useful if one wishes to view entire long bones with epiphysis, physis and metaphysis present in one specimen. If the cartilage matrix components are of interest, the metachromatic stains, including azure A and the critical electrolyte staining series with Alcian blue, are very valuable (Figure 3).¹¹ Periodic acid-Schiff can be applied both to decalcified and plastic embedded specimens to investigate the mucopolysaccharide content of cartilage.²¹

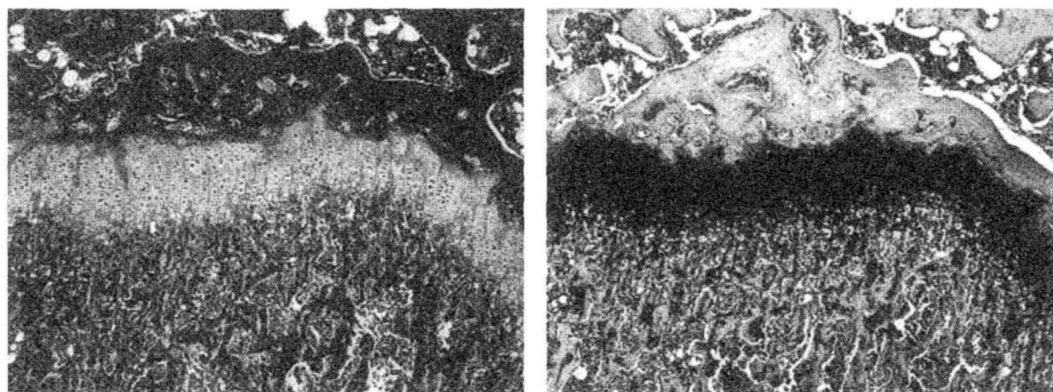


FIGURE 3. Photomicrographs illustrating differential staining of the growth plate of a decalcified rat femur obtained with Masson trichrome stain (left) (bone dark, growth plate pale) and with safranin-O (right) (bone pale, growth plate dark) ($\times 44$).

2. Undecalcified Sections

Staining regimes for plastics require modification because these procedures are usually performed without the removal of the plastic and thus different timing regimens are utilized. In Appendix 1 we list references for the staining procedures which we routinely carry out. Goldner's stain and von Kossa's stain both result in differentiation of osteoid from mature mineralized bone matrix; Goldner's stain (a modified Masson trichrome) has a hematoxylin component which provides excellent cellular staining.²¹ Cement lines, which are specialized regions of the bone matrix which mark past sites of bone remodelling, can be visualized with a number of staining procedures, including Stains-All,²² toluidine blue or methylene blue/basic fuchsin,¹⁶ thionine,²³ a combined method using thionine, toluidine blue, methylene blue chloride or methylene violet,¹⁶ or modifications of Bodian silver stain.¹⁷

Villanueva et al.²⁴ developed a method for block staining of the specimen followed by hand grinding. With other methods where specimens are not stained en bloc, only the surface of ground sections is stained. Bone embedded in plastic initially can also be cut, polished and stained. This may offer some advantage if small rodent long bones are being studied in cross section since the plastic margin makes the specimen easier to handle. Other useful suggestions in handling and staining ground sections have been summarized by Schenk et al.¹⁸

B. STAINING OF SOFT TISSUES

Soft tissue-bone interfaces can be examined using special staining methods to distinguish fibrocartilage, bone and soft tissues; Hurov has presented a battery of stains which help histologically define this specialized site (Verhoeff's elastic tissue stain, Alcian blue and periodic acid-Schiff for acid mucopolysaccharides, hematoxylin and eosin, and an aldehyde fuchsin counterstained with Van Gieson's picro-fuchsin to demarcate cartilage and bone).²⁵

C. HISTOCHEMICAL STAINING

Enzyme histochemistry has played an important role in the study of chondro-osseous tissue. The two phosphohydrolases with special relevance are alkaline phosphatase, an ectoenzyme present in the osteoblast and in matrix vesicle membranes, and tartrate-resistant acid phosphatase (TRAP), a lysosomal enzyme whose localization provides a sensitive method of osteoclast identification (Figure 4).

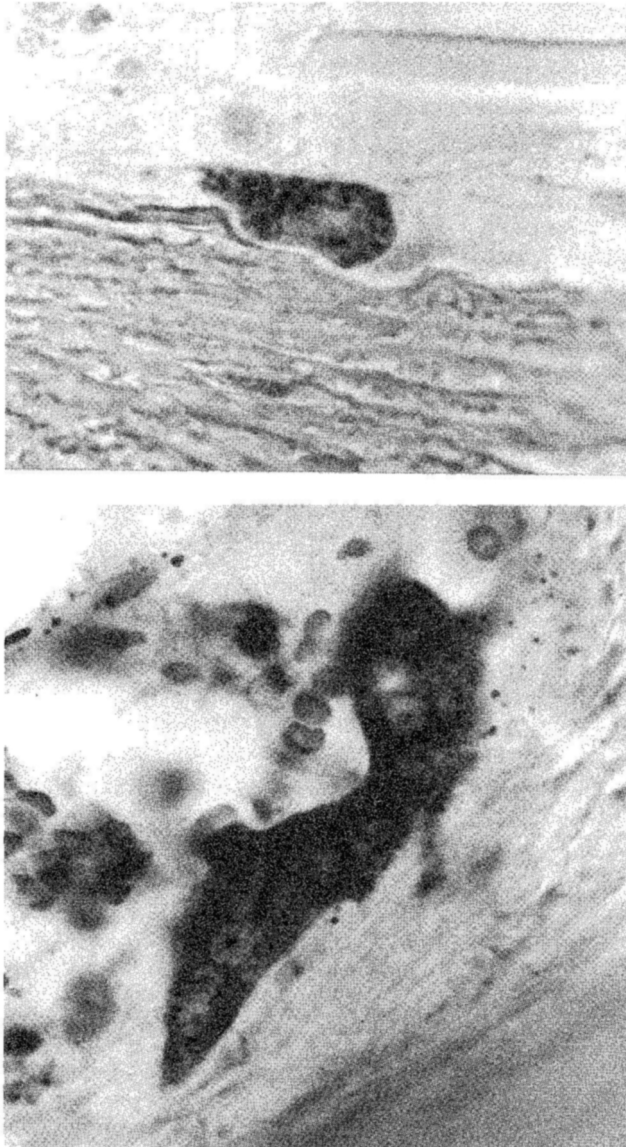


FIGURE 4. Localization of tartrate-resistant acid phosphatase (TRAP) is seen here in identification of both small (top) and large (bottom) osteoclasts. (methyl methacrylate undecalcified preparation, $\times 1080$). (From Gruber, H. E., et al., *Semin. Haematol.*, 18, 258, 1981. With permission.)

Three factors influence one's success in histologically localizing enzymes: optimum procurement of the specimen, proper fixation, and proper embedding. For bone and cartilage, the latter condition can be satisfied by using methacrylate embedding and avoiding decalcification and paraffin processing.

A critical factor is appropriate fixation. Results from the authors' laboratory have shown that optimum localization of acid phosphatase (Figure 4) and alkaline (Figure 5) in fresh tissue is achieved after fixation not exceeding 15 hours in neutral buffered formalin followed by embedment in methyl methacrylate.⁹ As a rule of thumb, 4–6 hours provides a safe fixation period. Occasionally, it is desirable to retrieve frozen tissue from -70°C collections. When frozen tissue is to be evaluated, good localization of both enzymes can be achieved after fixation for 30 min. and embedment in methyl methacrylate, or after five min. fixation followed by embedment in glycol methacrylate.

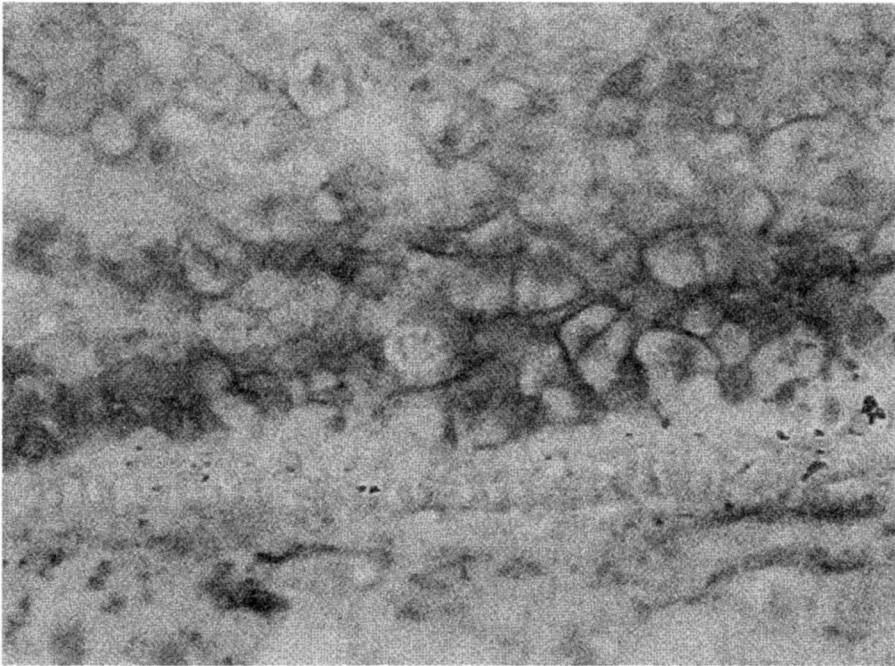


FIGURE 5. Localization of alkaline phosphatase in osteoblasts. (glycol methacrylate, $\times 420$).

Often cytochemical studies of many enzymes are done on minimally fixed tissue, often sectioned on a cryostat. This, however, requires considerable technical skill with fragile growth plate or brittle large bone specimens, so it is well worth the effort to define the appropriate fixation period for each enzyme of interest.

It is important to have a positive control included in all runs with alkaline phosphatase and TRAP localizations.

VII. HISTOLOGICAL EVALUATION AND HISTOMORPHOMETRY

Qualitative evaluation of histologic changes in bone and cartilage is usually the first level of evaluation of specimens from an orthopaedic experimental study. Initial qualitative studies are of value because they help focus the investigator on specific sampling sites of interest under the microscope and help to further refine the investigator's notion of what type of quantitative information should be collected in the study. For large projects involving many investigators, this is also a useful first approach to the histologic study of the specimens of interest whereby teams of researchers discuss, evaluate and agree on the quantitative data to be collected. Qualitative studies are also of value should experimental treatment produce biochemical changes in matrices which can be revealed by special staining techniques. Histology in such research can play a valuable role prior to biochemical tissue analysis.

For most well defined studies of bone and cartilage, however, current research leads to quantitative evaluation of specific features of the tissue of interest. The most common types of data collected are those measuring tissue areas (areas of bone, osteoid, and fibrous tissue in the marrow cavity), widths (trabecular width, osteoid seam width, growth plate widths, widths between tetracycline labels), lengths (the fraction of endosteal surface lined by osteoid, or with an associated tetracycline label), or counts (osteoblast or osteoclast indices). The advent of PC-based, interactive image analysis systems designed for the specialized requirements of bone histomorphometry have

and newer immunologic and non-immunologic visualization methods.^{27,28} For orthopaedic research purposes, the specimens are usually either fresh frozen and cut on a cryostat, or fixed appropriately as described below.

Since immunohistochemical methods are technically complex, most studies on bone have utilized decalcified preparations. There are, however, excellent studies using plastic embedded tissues which are best recommended to the more advanced histology laboratory.²⁹⁻³¹

As noted above for enzyme histochemical studies of bone, an initial step in attempting to use a new antibody localization method is to determine the type of fixation, if any, which is required. As noted by Myers,³² fixatives which maintain excellent morphologic detail in a tissue may not be at all successful in preserving immunoreactivity. Again, for the novice investigating an antibody for the first time, testing of fixation agents (1 or 10% neutral buffered formalin, Bouin's fixative, or Zamboni's fixative) is the first step. The reader is referred to detailed texts for further information about fixation types and regimes which may be of interest.^{27,28} A methodological necessity is inclusion of known positive control tissue, and conscientious inclusion of negative controls, with each assay run.

Other common problems encountered with immunohistochemistry are poor visualization resulting from insufficient specific staining, high levels of nonspecific (background) staining, the inability to achieve localization due to masking by prolonged fixation or embedding methods, and lack of success with initial trials of new antibodies. Thorough rinsing between steps is important to control non-specific staining. Some investigators have reported success in retrieving antigenicity in specimens fixed for inappropriately long periods (or older archived fixed tissue) with use of antigen retrieval methodologies as recently reviewed elsewhere.³³⁻³⁷ These methods include techniques such as enzyme pretreatment of sections and microwave irradiation.

There are two other common problems to be resolved when beginning new studies: the first is to identify reliable antibodies for your specific needs, and if the antibody is an anti-human one, to determine whether or not it can successfully be used to localize antigens in nonhuman tissue. Appendix 3 suggests some sources for information should routine literature searches and vendor product information be insufficient. It is important to remember that not all commercially available antibodies work equally well, and that there is no certification system for antibodies similar to that which ensures chemical histologic stain quality through the Biological Stain Commission and its issued certification numbers for stains.

B. *IN SITU* HYBRIDIZATION

This is a powerful technical procedure to localize either DNA or RNA nucleic acid sequences within cells, and as such gives the investigator the tool with which to visualize gene expression and other events at the cell level. This technological marriage between molecular biology techniques and techniques used for immunocytochemical imaging raises the complexity over each of the individual methods. Fixation and methods for pretreatment of sections must be optimized for each tissue and probe. It is also important to utilize a method wherein the reporter molecule in indirect procedures does not interfere with the hybridization reaction or the resultant hybrid stability.³⁹ The reader is referred to several excellent recent texts and laboratory manuals which discuss probe size, radioactive vs non-radioactive markers, and specialized strategies.³⁸⁻⁴⁰

In situ polymerase chain reaction (PCR) can be utilized to detect low-copy DNA and RNA; optimization is required for fixation, protease digestion, DNase digestion for RNA targets, amplification solution, and for DNA targets, the hot start procedure.⁴¹

An increasing number of *in situ* hybridization kits are becoming available for specific probes of interest,⁴⁰ and in our laboratory we routinely localize cells undergoing programmed cell death (apoptosis) with such a kit procedure (Figure 7).

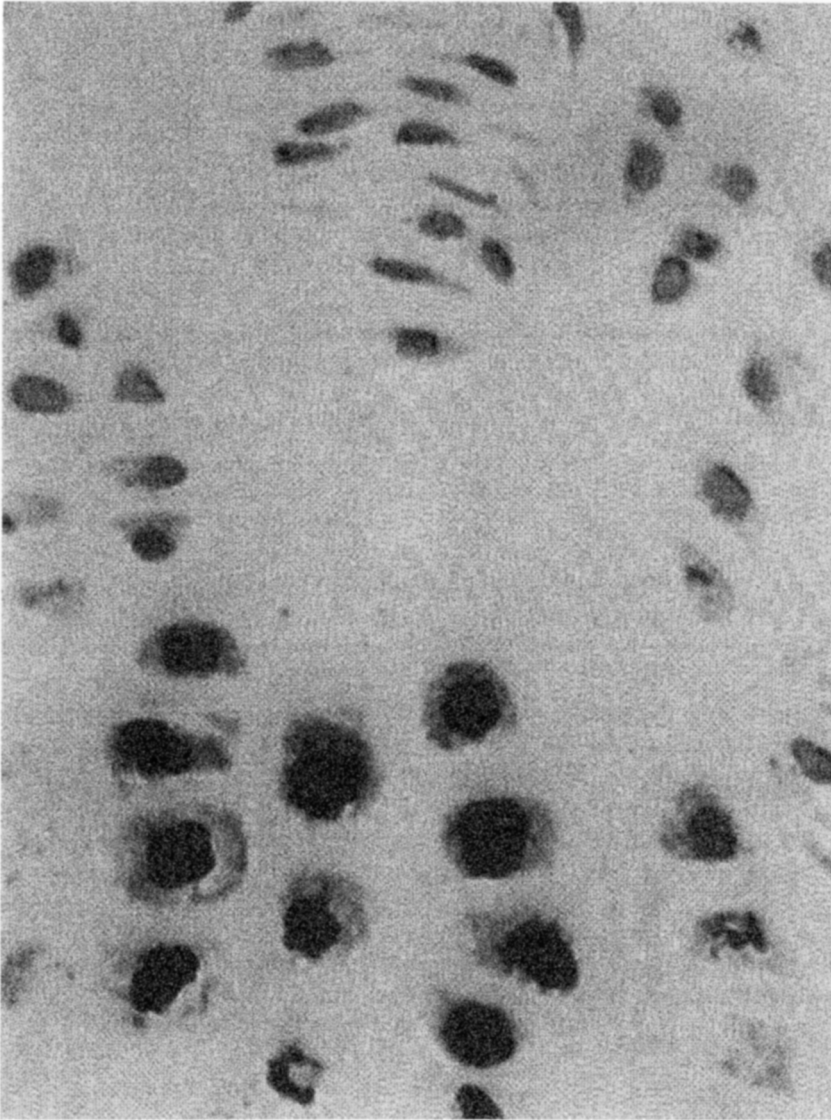


FIGURE 7. Photomicrograph illustrating *in situ* localization of apoptotic chondrocytes (darker cells) in the hypertrophic zone of the growth plate of a rat. (decalcified paraffin-embedded specimen, $\times 420$).

C. TISSUES CONTAINING IMPLANTS

The diversity of biomaterials employed as implants is expanding rapidly. Implants are studied in orthopaedic research both for their application in architectural reconstruction (to restore, maintain or improve function), to repair defects, and as drug or bioactive agent delivery systems. Both permanent and biodegradable implants are being utilized. Implant types vary greatly; implant materials may be metal, may be ceramic, may be coralline, may be plastic, may be solid or porous, surfaces may be polished or may be roughened or may be coated with other materials in an attempt to achieve a solid bone-implant interface. Both natural and synthetic polymers in many configurations (sheets, sponges, gels) are playing a role in investigations to explore tissue engineered growth of bone, cartilage and other soft tissues.⁴²

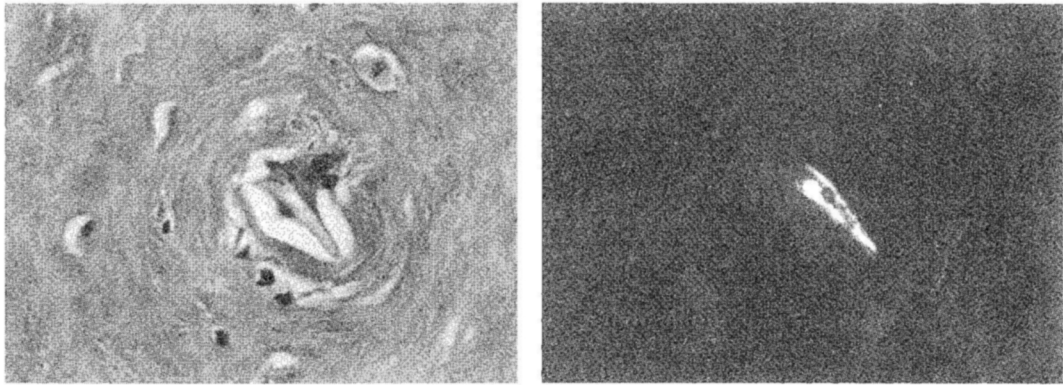


FIGURE 8. Photomicrographs illustrating wear particles in the fibrous capsule surrounding a deteriorating hip prosthesis in a patient with severe rheumatoid arthritis. Left, bright field microscopy shows a fibrous tissue region with a giant cell surrounding a possible wear particle; Right, the same microscopic field viewed with polarized light microscopy: the wear particle stands out well and is easily visualized because of its birefringent nature ($\times 420$).

The methods for general histologic examination of bone described above all have a role in the histologic examination of implant sites. Biodegradable or gel implant materials can be associated with mineralization *in situ* and require decalcification if paraffin embedding or plastic embedding as the method of choice. Porous implants of metal and metal alloys require diamond saw (or ceramic carborundum) blades for cutting and subsequent grinding and surface staining. Methacrylate infiltration and embedding help preserve the bone-implant interface. Mechanized grinding wheels may be required for large specimens with large metal implants.

As with all bone histologic studies, a sampling site must be identified clearly and the sections obtained in the appropriate plane with minimum artifact during gross specimen harvest. At tissue harvest, the gross features of nearby tissue reaction, encapsulation, or other tissue responses should be noted and photographically documented. Radiographic documentation at harvest is also often very important. Large specimens require the appropriate lengthened infiltration regimes for methacrylate embedding.

The histologic evaluation of implant specimens should include evaluation of any tissue reaction to the implant (tissue necrosis, inflammation, giant cells, neutrophils, macrophages, fibroblasts, fibrosis, encapsulation, scarring). For some implants, evaluations should include assessment of possible sarcoma formation around metal implants. The response of blood vessels near the implant, and ingrowth of vasculature into porous or biodegradable implants, is an important feature which can be monitored in microtome sections using anti-Factor VIII immunohistochemistry to denote blood vessels. For implant studies which need to assess new bone formation on or within the construct, undecalcified methods will allow assessment of the extent and quality of osteoid seams and tetracycline labelling evaluation if desired. Care should be taken to note the quality as well as quantity of new bone formed. Polarized light microscopy should be used to identify areas of poorly modelled, woven bone and well modelled lamellar bone. Polarized light microscopy is also valuable to detect wear debris in capsular material (Figure 8). With careful harvesting and preparative techniques, the interface between the implant and bone can be successfully preserved.

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APPENDIX 1

SELECTED PROTOCOLS FOR EMBEDDING AND STAINING

A. PARAFFIN EMBEDDING

1. Fixation

If enzyme histochemistry is desired, routine fixation should be in 10% buffered formalin for 4–6 hours with a vacuum pull until bubbles cease to emerge from the specimen. Immediately after placing the specimen in fixative, make a small identification tag with plastic paper and a lead pencil. This tag follows the specimen through all solution changes and into embedment, thus assuring correct specimen identification. The specimen can be transferred to, and held in, 70% ethanol.

2. Decalcification Methods

a. *EDTA Method*

EDTA solution

40 mg EDTA in 300 ml distilled H₂O (dH₂O).

Add 12.6 g NaOH pellets; add slowly, four pellets at a time.

The pH should be 7.3 when solution is prepared.

Place the specimen in a large volume of EDTA decalcifying solution (at least 4–5 times the volume of the specimen). Keep the specimen rotating or being stirred for several days. Rinse the specimen for 2–3 hours after decalcification. The specimen can be held in 70% ethanol for further processing. For small bones, decalcification may take 3–4 days. Change the solution at least once during this period. This method is useful for most types of immunohistochemistry.

b. *Formic-Citrate Method*

Solution A 50 g sodium citrate in 250 ml dH₂O.

Solution B 50 ml (90%) formic acid in 50 ml dH₂O.

Working solution Mix equal portions of Solution A and Solution B.

Decalcify in 4–5× the volume of the specimen. Place the specimen in a container on a shaker or rotator at room temperature. The size of the specimen will influence the length of time required for decalcification. A rat tibia requires approximately 4 hours. Rinse the specimen for 2–3 hours after decalcification. The specimen can be held in 70% ethanol for further processing.

B. METHYL METHACRYLATE EMBEDDING OF SMALL TO MEDIUM SIZE SPECIMENS

1. Preparation and Fixation of Small to Medium Size Specimens

Specimen should be sketched or photographed when obtained. A detailed description of the tissue should be kept when dissection begins.

Soft tissue that is not necessary for the analysis should be dissected off. Measurements of small bones can be taken prior to trimming by using a caliper. The trimmed piece should be placed in 10% buffered formalin for no more than 24 hours. An exception to this rule is the inclusion of acid phosphatase or alkaline phosphatase localization. In this case, specimens should be fixed for 4–6 hours. A paper label (plastic paper written in pencil) with the identification code should accompany the specimen throughout processing.

2. Dehydration and Infiltration Schedule for Small to Medium Size Specimens

After 24 hours, or the shorter fixation times, the tissue should be placed in 70% ethanol. A vacuum condition should be created until air bubbles cease to emerge from the specimen. Keep tissue in 70% ethanol until dehydration or for long term storage. Depending on the size of the specimen, use the guidelines listed in Appendix Table 1 for dehydration and infiltration:

APPENDIX TABLE 1

Dehydration and Infiltration of Small to Medium Size Specimens

Processing step*	Mouse bones	Rat tibia or fibula	Rat femur, vertebrae
95% ethanol	2–3 hours†	3–4 hours	4–6 hours
95% ethanol	Change, 2–3 hours	Change, 3–4 hours	Change, overnight
100% ethanol	Overnight	Overnight	Change, 4–6 hours
100% ethanol	Change in morning, 2–3 hours	Change in the morning, 6–7 hours	Change in the afternoon, cap, overnight
Working Solution A (1st infiltration)	Cap 2–3 days	Place in methyl in the afternoon, cap, 2–3 days	Place in methyl in the morning, cap, 3–4 days
Working Solution A (2nd infiltration)	Change, cap, 2–3 days	Change, cap, 3–4 days	Change, cap, 3–4 days

* See section C below for preparation of methacrylate solutions

† Vacuum condition is needed for every step.

3. Methyl Methacrylate Infiltration and Embedding

a. Infiltration

After dehydrating the specimen according to the protocols listed in the Appendix Table 1, place the specimen into the Working Solution A described in Section C below and add 0.9 gm/100 ml benzoyl peroxide. This solution is the infiltration solution. All infiltrations are carried out in either a cold room or refrigerator. Vacuum applications must be performed under a vented fume hood.

b. Embedding

Embedding solution

100 ml of Working Solution A

0.9 gm of benzoyl peroxide

1 ml of JB-4 Solution B (from the JB-4 kit supplied by Polysciences)

Choose a size of embedding mold appropriate for the size of the specimen. Orient the specimen so that the crucial side is on the bottom, then pour the embedding solution over the specimen. Insert a label (made with plastic paper and a No. 2 pencil). Place an aluminum embedding block carefully over the specimen. Try not to move the mold while doing this. Add more embedment solution to the specimen so the level of solution is surrounding the aluminum block holder.

Carefully transfer the molds to a vacuum desiccator to remove any excess air bubbles. Pull a vacuum for 5–10 minutes. Release the vacuum gently and transfer the molds to a GasPak container and seal it off. The oxygen has to be removed to allow polymerization to occur. Flush the GasPak with nitrogen gas for 5–10 minutes and close off the tubing without letting any oxygen in. The rate of flow from the nitrogen tank should be fairly slow so it does not dry out the specimens and is maintained continuously to allow the removal of the air. After flushing, seal the container and leave it undisturbed overnight at room temperature. The polymerization process should be complete in less than 16 hours.

Open the GasPak under a fume hood and remove the molds. Allow the specimens to air out under the fume hood for a few hours. The specimens are now ready to be sectioned on the Leica 2065 microtome or Polycut E microtome with a tungsten carbide knife.

Cautions Avoid all skin contact with methyl methacrylate. Take extreme caution not to inhale fumes from methacrylate solutions. Gloves, lab coats, goggles, and a vented fume hood should be used when handling these solutions.

4. Sectioning the Methyl Methacrylate for Small to Medium Size Specimens

Remove excess methacrylate from the block with a hacksaw, bandsaw, hand-held small trimming saw (Dremel), or a Histosaw. Only a small amount of methyl methacrylate is needed to be left around the specimen.

Before sectioning, make sure the blade is securely set in the knife holder and the specimen is locked in place. Never wear any loose clothing or jewelry when sectioning. Keep fingers away from the knife. Leave guard in place when stepping away from microtome or changing the orientation of the block.

To set up the microtome, face off the block with a trimming knife. Face off the block at the area of interest and then change knives to take the good sections.

The sections are placed in 6, 12, 24, or 48 well tissue culture plates depending on the size of the section. Collect four sections per well and continue collecting adjacent sections to fill 6–12 wells. This is dependent upon which staining protocols will be performed and how many sections are kept in reserve. If the sample has been labelled with tetracycline, save any sections adjacent to the measuring sections for tetracycline analysis.

Methyl methacrylate sections can be stained free-floating, dried, and coverslipped using Permount (Fisher).

C. PREPARATION OF METHACRYLATE SOLUTIONS

1. Removal of Inhibitors from Methacrylate Solutions

Use basic alumina AG-10 for column filtration to remove commercially added inhibitors from solutions to be used in infiltration and embedding.

a. *Methyl Methacrylate*

Mount a column (use a narrow stem one with a funnel placed in it) on a ring stand in the fume hood. Fill the column 3/4 full with basic alumina. Fill the column with methyl methacrylate and allow it to filter through the alumina and be collected in a container. Do not allow the column to run dry. If it is stored, place a clamped rubber tube at the bottom of the column. Store filtered solution in a refrigerator for no longer than six months. Discard the alumina in the column when it turns grey blue. This means it can no longer act chemically to remove the inhibitors.

b. *Glycol Methacrylate (2-hydroxyethyl methacrylate)*

Shake the glycol methacrylate and basic alumina together and store tightly capped in the refrigerator. Shake frequently. Filter or centrifuge when needed for infiltration solution preparation. Store filtered solution in a refrigerator for no longer than six months. Discard the alumina when it turns grey blue; this means it can no longer act chemically to remove the inhibitors.

Cautions Avoid all skin contact with methyl methacrylate. Take extreme caution not to inhale fumes from methacrylate solutions. Gloves, lab coats, goggles, and a vented fume hood should be used when handling these solutions.

2. Preparation of Methyl Methacrylate Solutions

Working Solution A

- 85 ml methyl methacrylate
- 10 ml glycol methacrylate
- 5 ml dibutyl phthalate
- 5 gm polyethylene glycol (PEG 600)

Heat mixture gently on a stirred hot plate until PEG is dissolved. Stir covered. Let solution cool to room temperature, place in glass container well covered and store in refrigerator. Solution is good for six months.

D. METHYL METHACRYLATE EMBEDDING OF THE LARGE SPECIMEN

For our large specimen methodology, see Gruber and Stasky¹⁷ for a procedure which employs increased dehydration intervals and use of a heat sink for polymerization in cooler temperatures.

E. GLYCOL METHACRYLATE PROTOCOLS FOR SMALLER BONES AND CARTILAGE

1. Infiltration

This methodology utilizes the JB-4 kit provided by Polysciences. Note that this method does not use serial ethanol dehydration of tissues. After fixation, the infiltration schedule is carried out as the steps listed in Appendix Table 2. Be sure that specimens are well capped during processing.

APPENDIX TABLE 2
Glycol Methacrylate Infiltration Schedule*

Processing step	Solution	Time/condition
1	70:30 JB-4 solution A:dH ₂ O	1 hr/shaker in cold room
2	85:15 JB-4 solution A:dH ₂ O	2–3 hours/shaker in cold room
3	95:5 JB-4 solution A:dH ₂ O	2–3 hours/shaker in cold room
4	10ml JB-4 Solution A + 0.09g JB-4 catalyst	2–3 days/shaker in cold room

* See section C for preparation of methacrylate solutions.

2. Embedding

Materials needed 5 ml Solution A + 0.1 ml Solution B + 0.045 gm JB-4 catalyst. Mix the catalyst into solution A until completely dissolved. Add Solution B, mix for one minute and start the embedding procedure. Put the solution, specimen and ID label in a mold with care to properly orient the specimen. Place the block holder on the mold, add additional solution, and seal the edges with melted paraffin wax. Polymerization will be complete at room temperature in 4–5 hours; let sit overnight for best results. Successful embedding produces blocks with no bubbles formed during polymerization.

Cautions Avoid all skin contact with methacrylates. Take extreme caution not to inhale fumes from methacrylate solutions. Gloves, lab coats, goggles and a vented fume hood should be used when handling these solutions.

3. Sectioning Guidelines for Glycol-embedded Specimens

Large amounts of excess methacrylate should be trimmed from the specimen but a sufficient margin of plastic left to support the specimen during sectioning. Trimming can be done with a hacksaw, Dremel, or Histosaw. Avoid contact of dust with skin.

Use an older trimming knife to face off the specimen down to the area of interest and then change to a good sectioning knife. Critical to good sections is a sharp knife edge; always use one site for only sectioning one specimen.

Glycol sections are collected and floated in a staining dish filled with room temperature dH₂O. Sections are collected on a slide and dried on a slide warmer. Toluidine blue provides a rapid screening stain to ensure that the region of interest has been reached in the specimen block. Glycol sections after staining are coverslipped using Pro-Texx Mounting Medium (American Scientific Products).

F. STAINING AND ENZYME LOCALIZATIONS

See references provided in the main body of this chapter for excellent surveys of staining procedures for bone and cartilage, such as Goldner's stain,²¹ alkaline and acid phosphatase enzyme localization,¹³ or cement line visualization.^{16,17,22}

APPENDIX 2

USEFUL EQUIPMENT AND TOOLS FOR THE ORTHOPAEDIC HISTOLOGY LABORATORY

Adhesive for binding large specimens to molds and rings prior to sectioning Technovit 3040 glue: Energy Beam Sciences, Agawam, MA; Heraeus Kulzer GmbH, Philipp-Reiss-Strasse 8/13, D-61273, Wehrheim, Germany.

Bone histomorphometry OsteoMeasure: OsteoMetrics, Inc., 2103 N. Decatur Road, Suite 140, Atlanta, GA, Tel: 404-876-1004; Fax: 404-876-4004; email: support@osteometrics.com. Provides both software and hardware support.

Diamond circular saw Isomet Low Speed Saw: Buehler Ltd., Lake Bluff, IL.

Diamond wire saws Well type 3241 Precision Wire Saw: Delaware Diamond Knives, Wilmington, DE.

Dyes for marking tissue orientation Marking dyes: Triangle Biomedical Sciences, Durham, NC.

Embedding rings for large specimens Histoprep embedding ring: Fisher Scientific.

Hand-held small trimming saw Dremel Model 732: Stoelting Instruments, Wooddale, IL.

Also a source for diamond-coated cutting discs for the Dremel.

Megacassettes for embedding large specimens Surgipath Medical Instruments, Richmond, IL.

Microtomes — Small to medium sized specimens Jung RM2065 microtome: Vashaw Scientific, Norcross, GA; *Large specimens*: Leica Polycut E.

Microtome knives Delaware Diamond Knives, Wilmington, DE. This vendor also resharpenes used knives. For undecalcified sectioning, tungsten-carbide knives are required. Small to medium sized specimens: 30° angle; large specimens: 50° angle.

Vacuum pump Gast Manufacturing Corp, Benton Harbor, MI, Model No. IHAB-25 MIOOX.

APPENDIX 3

ON-LINE HELP AND OTHER SOURCES OF INFORMATION

histonet@pathology.swmed.edu An email based source for placing questions which other users can answer on line.

<http://www.antibodies.probes.com/> A web page which gives instructions on how to subscribe to this service which is a source for antibody information.

boneworld@osteometrics.com An email based source for asking questions about bone histology; free; interested parties can subscribe; questions are automatically broadcast to all members and can respond. Subscribe at: subscribe@osteometrics.com

Linscott's Directory of Immunological and Biological Reagents. Available both in soft bound and diskette formats. Address: 4877 Grange Road, Santa Rosa, CA 95404, Tel: (707) 544-9555; Fax: (415) 389-6025. Useful information/sources with cross-indices for mono- and poly-clonal antibodies, kits, and other data.

8 Mechanical Properties and Testing Methods of Bone

Yuehuei H. An and Robert A. Draughn

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I. INTRODUCTION

Mechanical properties are basic parameters which reflect the structure and function of bone. The mechanical properties of bone obey Newton's and Hook's laws of mechanics.¹ The mechanical behavior of bone in normal physiological situations is similar to that of an elastic material. However, unlike inorganic materials, bone has the ability to repair itself and can alter its mechanical properties and morphology in response to increased or decreased function.

Mechanical testing of bone is involved in most bone-related animal studies. A survey of articles published in the *J. Orthop. Res.* in 1995 revealed that in 77% (23/30) of bone-related animal studies mechanical tests were utilized or biomechanical principles or theories were employed.

Because most orthopaedic surgeons and many basic science researchers are not well versed in biomechanics and help from an expert is not always available, this chapter emphasizes basic biomechanical theories and how to do common mechanical tests.

II. COMPOSITION AND STRUCTURE OF BONE

Bone is a composite material which consists of organic matrix (mainly collagen) and inorganic hydroxyapatite. Water accounts for about 20% of the wet weight of cortical bone, hydroxyapatite (HA) makes up approximately 45%, and the organic substances (90–95% collagen, 1% glycosaminoglycans, 5% other proteins) account for the remaining 35%. The presence of mineral is responsible for the strength and hardness of bone.

Bone structure can be observed at several levels:² (1) At the most fundamental level, HA crystals are embedded between the ends of adjoining collagen fibrils. This composite of rigid HA and flexible collagen provides a material that is superior in mechanical properties to either of them alone. Bone is more ductile than hydroxyapatite, allowing the absorption of more energy before failure, and more rigid than collagen, permitting greater load bearing. (2) At the second level, the collagen-HA fibrils are formed into sheets or lamellae with a preferred direction. The orientations of the fibers define directions of maximum and minimum strengths for a primary loading direction. (3) The third level consists of the arrangement of the lamellae. A circular concentric structure produces a tubular Haversian osteon with maximum strength along its long axis. Alternately, lamellae may be arranged in sheets, as found in plexiform bone, in which case the strength of the material transverse to the longitudinal axis is lower in the direction in which lamellae are being pulled apart at cement lines and higher in the plane of the sheets. (4) The fourth level of structure is the macroscopic materials, cortical or trabecular bone. The main factors determining strength at this level are the density of the bone and trabecular orientation.

Good articles on bone structure and composition and their relation to mechanical properties were presented by Carter and Spengler,³ Katz,⁴ Hoesler,⁵ Cowin,¹ Tencer,² Hayes and Bouxsein.⁶

III. MECHANICAL PROPERTIES OF BONE

A. MECHANICAL PARAMETERS

Bone has two main mechanical properties: stiffness and strength. Stiffness is expressed by the elastic modulus which is a measure of the stress required to elastically deform the bone. Strength defines the stress required to fracture the bone. Measures of stiffness and strength need to be recorded and calculated during a mechanical test. If the testing is used for comparing differences between groups, directly recorded values of stiffness and ultimate load are adequate for property evaluation. If the purpose of the study is comparing differences between values generated from different laboratories or from different experimental settings, elastic modulus and strength must be calculated to provide standardized values of stiffness and load.

The above-mentioned mechanical parameters are based on load-displacement curve which is recorded by a chart recorder or computer. The curve gives the yield load, ultimate load, deformation, and stiffness directly (Figure 1). Since the dimensions of specimens are readily measured, the values of elastic modulus and strength are easily calculated.

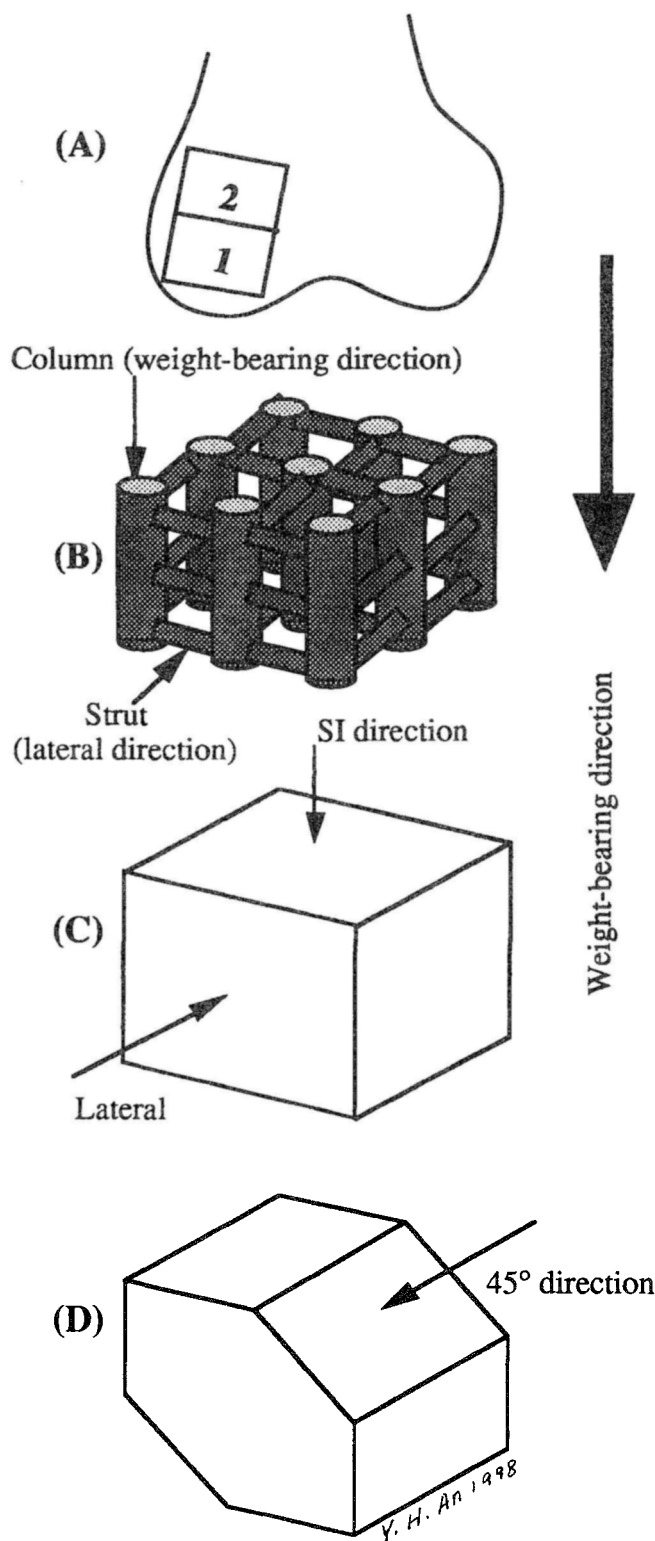


FIGURE 1. A test of screw-holding power of bovine cancellous bone from different directions. (A) represents sampling site and orientation. In (B) the structure of the bone block is idealized into a column-strut model. The columns indicate thicker trabeculae in the weight-bearing direction. The struts represent the trabecular connections between columns (column trabeculae). It is assumed that columns are thicker and stronger and struts are thinner and weaker. (C) and (D) show directions of screw insertions.

TABLE 1**Bending properties of human and animal cortical bones (selected data from the literature)**

Species	Bone	Specimen	Mechanical testing	Strength (MPa)	Elastic modulus(GPa)	First author, year ^{Ref.}
Human	Femur	2×5×350 mm beam	3 pt. bending	181	15.5	Sedlin 1966 ⁷
		3×3×30 mm beam	4 pt. bending	103–238*	9.82–15.7*	Keller 1990 ⁸
		0.4×5×7 mm beam	3 pt. bending	225 ± 28	12.5 ± 2.1	Lotz 1991 ⁹
		2.0×3.4×40 mm	3 pt. bending	142–170*	9.1–14.4*	Currey 1997 ¹⁰
Monkey	Tibia	Whole bone	3 pt. bending	—	9.0 ± 1.3	Kasra 1994 ¹¹
Cattle	Femur	2×3.5×30 mm beam	3 pt. bending	—	18.5 ± 2.8	Currey 1988 ¹²
		2×4×35 mm beam	3 pt. bending	228 ± 5	19.4 ± 0.7	Currey 1988 ¹³
		23×0.4 mm beam	3 pt. bending	209 ± 13	18.1 ± 0.5	Currey 1995 ¹⁴
	Tibia	4×4×35 mm beam	Bending	—	14.1	Simkin 1973 ¹⁵
Horse	Femur	4×10×80 mm beam	3 pt. bending	230 ± 18	21.0 ± 1.9	Martin 1993 ¹⁶
		2×2×40 mm beam	4 pt. bending	204–247*	17.1–19.9*	Schryver 1978 ¹⁷
		2×3.5×30 mm beam	3 pt. bending	—	21.2 ± 1.9	Currey 1988 ¹²
	Radius	2×2×40 mm beam	4 pt. bending	217–249*	16.2–20.2*	Schryver 1978 ¹⁷
	Metacarpus	2×2×40 mm beam	4 pt. bending	226–240*	17.0–18.4*	Schryver 1978 ¹⁷
Sheep	3MT, 3MC†	1.8×4.5×70 mm	4 pt. bending	195–226*	14–16*	Bigot 1996 ¹⁸
	Metacarpus	2×3.5×30 mm beam	3 pt. bending	—	18.9 ± 2.2	Currey 1988 ¹²
	Donkey	Radius	2×3.5×30 mm beam	3 pt. bending	—	17.6 ± 2.0
Dog	Humerus	Whole bone	3 pt. bending	193 ± 35	2.7 ± 0.6‡	Kaneps 1997 ¹⁹
Pig	Femur	Whole bone	3 pt. bending	39.9	0.37‡	Crenshaw 1981 ²⁰
	Rib	Whole bone	3 pt. bending	35.6	2.24‡	Crenshaw 1981 ²⁰
	3MC†	Whole bone	3 pt. bending	37.2	0.22‡	Crenshaw 1981 ²⁰
Goose	Femur	1.0×25 mm beam	3 pt. bending	232–283*	16.9–20.7*	McAlister 1983 ²¹
Cat	Femur	Whole bone	3 pt. bending	36 ± 9.47	7.1 ± 0.9	Ayers 1996 ²²
	Tibia	Whole bone	3 pt. bending	60.5 ± 12	11.4 ± 3.2	Ayers 1996 ²²
Rabbit	Femur	Whole bone	3 pt. bending	130 ± 5	13.6 ± 0.4	An 1996 ²³
		Whole bone	3 pt. bending	88 ± 20	10.7 ± 2.5	Ayers 1996 ²²
	Tibia	Whole bone	3 pt. bending	195 ± 6	21.3 ± 0.7	An 1996 ²³
		Whole bone	3 pt. bending	192 ± 47	23.3 ± 7.0	Ayers 1996 ²²
Rat	Humerus	Whole bone	3 pt. bending	167 ± 5	13.3 ± 0.6	An 1996 ²³
	Femur	Whole bone	3 pt. bending	180 ± 6	6.9 ± 0.3	Jørgensen 1991 ²⁴
		Whole bone	3 pt. bending	134 ± 4	8.0 ± 0.4	Barengolts 1993 ²⁵
Mouse	Femur	Whole bone	3 pt. bending	153 ± 45	4.9 ± 4	Ejersted 1993 ²⁶
		Whole bone	3 pt. bending	104–173*	8.8–11.4*	Simske 1992 ²⁷
	Tibia	Whole bone	3 pt. bending	40 ± 13	5.3 ± 1.8	Ayers 1996 ²²
		Whole bone	3 pt. bending	78 ± 12	8.9 ± 0.2	Ayers 1996 ²²

* Range of average values; †Third metatarsus and third metacarpus; ‡Value is questionable.

B. MECHANICAL PROPERTIES OF CORTICAL BONE

The dense nature of cortical bone determines its strong and stiff mechanical properties compared to cancellous bone. The mechanical properties of cortical bone depends on the type of mechanical testing. Although the tensile test is the standard method for testing mechanical properties of cortical bone, bending tests are used the most often (Table 1). The bending strength and elastic modulus of cortical bone ranges from 35 to 283 MPa and from 5 to 23 GPa (excluding the values marked with ‡) respectively. The strength and elastic modulus by tensile and compression tests ranges from 92 to 295 MPa and from 7 to 34 GPa respectively (Table 2). The tensile strength is about 2/3 of

TABLE 2

Mechanical properties of human and animal cortical bones tested by compression, tensile, and torsional testing (selected data from the literature)

Mechanical test	Species	Bone	Specimen dimensions	Strength (Mpa)	Elastic modulus(GPa)	First author, year ^{ref.}		
Compression	Human	Femur	2×2×6 mm dumbbell	167–215*	14.7–19.7*	Reilly 1974 ²⁸		
			2×2×6 mm dumbbell	179–209*	15.4–18.6*	Burstein 1976 ²⁹		
			3 mm dia. cylindrical dumbbell	205–206*	—	Cezayirlioglu 1985 ³⁰		
		Tibia	2×2×6 mm dumbbell	183–213*	24.5–34.3*	Burstein 1976 ²⁹		
			3 mm dia. cylindrical dumbbell	192–213*	—	Cezayirlioglu 1985 ³⁰		
			Bovine	Femur	3.8×2.3×76 mm dumbbell	133	24.1–27.6*	McElhaney 1964 ³¹
	Tensile	Human	Femur	2×2×6 mm dumbbell	240–295*	21.9–31.4*	Reilly 1974 ²⁸	
				Tibia	4×5 mm rectangular	165	23.8 ± 2.2	Simkin 1973 ¹⁵
				2×2×6 mm dumbbell	228 ± 31	20.9 ± 3.26	Reilly 1974 ²⁸	
			3 mm dia. cylindrical dumbbell	217 ± 27	—	Cezayirlioglu 1985 ³⁰		
			Goose	Femur	0.8 mm dia./2.4 mm cylinder	164–203*	12.2–14.6*	McAlister 1983 ²¹
			Torsional	Human	Femur	3.8×2.3×76 mm dumbbell	66–107*	10.9–20.6*
		2×2×6 mm dumbbell				107–140*	11.4–19.7*	Reilly 1974 ²⁸
2×2×6 mm dumbbell		120–140*				15.6–17.7*	Burstein 1976 ²⁹	
3 mm dia. cylindrical dumbbell		133–136*			—	Cezayirlioglu 1985 ³⁰		
Tibia		2×2×6 mm dumbbell			145–170*	18.9–29.2*	Burstein 1976 ²⁹	
		1.7×1.8×25 mm deam			162 ± 15	19.7 ± 2.4	Vincentelli 1985 ³³	
		3 mm dia. cylindrical dumbbell		154–158*	—	Cezayirlioglu 1985 ³⁰		
Bovine		Femur		3.8×2.3×76 mm dumbbell	92	20.5	McElhaney 1964 ³¹	
	2×2×6 mm dumbbell			129–182*	23.1–30.4*	Reilly 1974 ²⁸		
	3 mm dia. cylindrical dumbbell			162 ± 14*	—	Cezayirlioglu 1985 ³⁰		
	Tibia	4×5×30 mm dumbbell		136	7.1 ± 1.1	Simkin 1973 ¹⁵		
		2×2×6 mm dumbbell		152 ± 17	21.6 ± 5.3	Reilly 1974 ²⁸		
		2×2×6 mm dumbbell		188 ± 9	28.2 ± 6.4	Burstein 1975 ³⁴		
		2×2×6 mm dumbbell	188 ± 9	28.2 ± 6.4	Burstein 1975 ³⁴			
Torsional	Human	Femur	?	53	—	Hazama 1964 ³⁵		
			?	54 ± 0.6	3.2	Yamada 1970 ³⁶		
			2×2×6 mm dumbbell	—	3.1–3.7*	Reilly 1974 ²⁸		
		3×3×6 mm dumbbell	65–71*	—	Reilly 1975 ³⁷			
		3 mm dia. cylindrical dumbbell	68–71*	—	Cezayirlioglu 1985 ³⁰			
		3 mm dia. cylindrical dumbbell	66–71*	—	Cezayirlioglu 1985 ³⁰			
	Bovine	Femur	3×3×6 mm dumbbell	62–67*	—	Reilly 1975 ³⁷		
			3 mm dia. cylindrical dumbbell	76 ± 6	—	Cezayirlioglu 1985 ³⁰		
		Tibia	3×3×6 mm dumbbell	62–67*	—	Reilly 1975 ³⁷		
			3 mm dia. cylindrical dumbbell	76 ± 6	—	Cezayirlioglu 1985 ³⁰		

* Range of average values from different subjects.

compression strength. The torsional strength is about 60 MPa in average. The torsional (shear) strength is approximately 1/3 to 1/2 of the values of the longitudinal modulus (tested by bending, tensile or compressive tests) (Table 2). And the torsional (shear) modulus is about 1/5 of the longitudinal modulus. The mechanical properties of cortical bone also depend on loading directions of the testing method. The longitudinal (normally the weight bearing direction) elastic modulus is about two times that of the transverse (lateral directions) elastic modulus.^{6,38}

C. MECHANICAL PROPERTIES OF CANCELLOUS BONE

The porous nature of cancellous bone, with bony trabecular columns and struts and marrow-filled pores or cavities (a two phase structure³⁹), lends itself to a mechanical description by both structural and material properties.

1. Structural Properties of Cancellous Bone

The structural properties of cancellous bone are commonly measured by compression, tensile, or bending tests. The common phrase “mechanical properties of cancellous bone” means the structural properties. It is known that the strength and elastic modulus by tensile tests are smaller than that by compression tests. For example, the strength by tensile test is approximately 60% of the value by compression test reported by Kaplan et al.,⁴⁰ and the elastic modulus by tensile test is approximately 70% of the value by compression test reported by Keaveny et al.^{41,42} The mechanical properties of cancellous bone depend on anatomic location and function. According to the data list summarized by Goldstein (21 sets of data generated using compression test), the average values of strength and elastic modulus of human cancellous bone from different locations (femur, tibia, humerus, radius, vertebrae, and iliac crest) are 6.6–36.2 MPa and 130–1080 MPa respectively.⁴³ According to the selected data from the literature (Table 3), the values of strength and elastic modulus of cancellous bone are 1.5–38 MPa and 10–1570 MPa respectively. The structural properties of cancellous bone are much smaller than those of cortical bone. The average value of elastic modulus is several hundred MPa for cancellous bone,⁴³ compared to 5–21 GPa for cortical bone.¹²

Several investigations have addressed the orthogonal mechanical properties of cancellous bone of both human and animals.^{52,61,62} The strength and elastic modulus of cancellous bone depend on the direction of the load employed, as normally measured at SI (superior-interior), AP (anterior-posterior), or ML (medial-lateral) directions. Ciarelli et al.⁶² found the highest overall mean of elastic moduli of human long bone metaphyseal locations to be in the SI direction, which is about 2.5 times the value at the AP direction. The AP direction is higher than that of the ML direction. An earlier study using vertebral cancellous bone specimens by Galante et al.⁶¹ also showed a similar pattern. In a recent study in the authors’ laboratory, it was found that the screw pullout strength of bovine cancellous bone also depends on the direction of the screw insertion (loading direction). The strength was strongest (55 ± 5 MPa) at the SI direction (0 degree), weakest (37 ± 5 MPa) at lateral direction (90 degree), and was intermediate (43 ± 4 MPa) at a direction of 45 degrees.⁶³ This phenomenon may be explained by a column-strut model proposed in this study (Figure 1).

The strength and stiffness of cancellous bone also varies in different epiphyseal-metaphyseal locations both in human⁶² and animals. In the authors’ laboratory, the strength and elastic modulus of epiphyseal-metaphyseal bones of animals, such as rats,⁶⁰ rabbits,²³ dogs,^{53,64} and goats⁶⁴ have been investigated using compression and indentation tests. Generally, for both humans and animals the cancellous bones of lower limbs (hind limbs) are stronger and stiffer than those of upper limbs (front limbs).

2. Material Properties of Cancellous Bone

The material properties of cancellous bone are defined by the intrinsic properties of individual trabeculae, which have been measured by mechanical testing of single trabeculae using methods such as buckling analysis,⁶⁵ compression test,⁶⁶ microtensile test,⁶⁷ cantilever test plus finite element modeling,⁶⁸ finite element modeling,⁶⁹ or ultrasound methods.⁶⁷ The elastic modulus of trabecular bone material (individual trabeculae) is less (10–30%) than that of cortical bone. For example, the elastic modulus is 14.8 GPa for trabeculae and 20.7 GPa for cortical bone measured by an ultrasonic technique and 10.4 GPa and 18.6 GPa respectively using a microtensile test.⁶⁷

Articles on the mechanical properties of animal cancellous bones include studies of bovine, canine, or goat distal femur, proximal tibia, and vertebrae determined by compression test (Table 3), or of canine, rabbit, or rat epiphysometaphyseal bones examined using indentation test.^{53,60,70}

IV. GENERAL CONSIDERATIONS OF MECHANICAL TESTING OF BONE

Because of the limited size and inhomogeneity of bone, accurate measurement of its mechanical properties, especially elastic modulus (fortunately, it is not always needed for comparing properties

between groups), is a challenging endeavor. The difficulty of standardizing technical procedures (sampling and testing) of mechanical testing is the main reason for the large differences among the reported data.⁷¹ Also, many intrinsic and extrinsic factors have tremendous influence on the mechanical properties. The investigator should always be aware of factors which may be involved in the determination of mechanical properties of bone.^{2,7,72}

A. SPECIMEN HARVESTING AND STORAGE

Bone specimens to be used for mechanical testing should be harvested with sufficient extra tissue around the area of interest. A hand saw or wire saw is efficient for cutting bone. Keeping surrounding soft tissue (muscle, fascia, or skin) intact is very helpful for protecting the bone from drying. Preparation of samples for testing always should be done immediately before mechanical testing.

The common method for storing bone specimens is freezing at -20°C . The bones should be wrapped in paper or cloth towels, immersed in saline and placed in an air-tight plastic bag. Thawing always should be done in saline. The effects of storage on mechanical properties of bone at -20°C for short periods of time are minor. The maximum effect reported is a 4.6% reduction of torsional strength of canine long bones.⁷³ However, after thawing, enzymes such as collagenases and proteases may become active and degrade the tissue. Also, enzymatic degradation is not completely arrested at -20°C .⁷⁴ With concerns about the effects of enzymes⁵⁴ and evaporation,⁷⁵ a question arises if there are significant effects of long term storage at -20°C . Panjabi et al.⁷⁶ found no significant effects of freezing for 7–8 months on the mechanical properties of human vertebrae bone. Roe et al.⁷⁷ found that bones frozen at -20°C for eight months did not become significantly weaker. Because time periods longer than eight months have not been reported for frozen storage at -20°C , storage at this temperature for more than eight months is not recommended. Alternatively, -70°C , -80°C , or even lower temperatures or liquid nitrogen are suggested for long term bone storage, since these temperatures may minimize evaporation⁷⁵ and markedly reduce enzyme activity.⁵⁴

Owing to complexity of an experiment or unforeseen circumstances, sometimes a specimen must be thawed and frozen multiple times. The question arises whether multiple freezing and thawing is harmful to the mechanical properties. This question has been partially answered by Linde and Sørensen,⁷⁸ who found that freezing and thawing five times did not alter the compressive properties of cancellous bones. In our recent study, the proximal portion of the tibia of adult cows was sectioned to produce bone slices. They were then subjected to four freezing-thawing conditions: freezing with and without saline solution, then thawing in saline solution or exposed to the air. The mechanical properties of the bone before and after the treatments (5 cycles of freezing and thawing) were measured using an indentation test. It was found that there is no significant effect on the ultimate load and stiffness of the bone. Only a slight difference was noticed for the specimens frozen without saline soaking and thawed in air.⁷⁹ This work supports the practice to freezing and thawing bone specimens in saline solution.

B. SAMPLE PREPARATION

Rough cuts can be made with a regular bandsaw equipped with a 1/4-inch fine tooth saw blade. To prevent burning, low speed should be used with sufficient saline irrigation. This kind of cutting may only affect 1 mm depth of bone at the surface, which can be ground off using a polishing wheel. Finer cuts can be made using an ultra fine jigsaw. A diamond wafering or diamond wire saw (Histosaw, Delaware Diamond Knives, Wilmington, DE) is particularly good for making smooth, parallel cuts. For fabrication of cylindrical samples, a table top drill press is sufficient for relatively large samples (>7 mm diam.). For 4–5 mm diameter samples, a lathe or milling machine is recommended.

TABLE 3
Mechanical properties and densities of human and animal cancellous bones (selected data from the literature)

Species	Bone	Specimen	Ultimate strength (MPa)	Elastic modulus (MPa)	Apparent density (gm/cm ³)	Ash density (gm/cm ³)	First author, year ^{Ref.}
Human	Femoral head	8 mm diam. cylinder	9.3 ± 4.5	900 ± 710	—	—	Martens 1983 ⁴⁴
	Proximal femur	8 mm diam. cylinder	6.6 ± 6.3	616 ± 707	—	—	Martens 1983 ⁴⁴
	Distal femur	8 mm cube	5.6 ± 3.8	298 ± 224	0.43 ± 0.15	0.26 ± 0.08	Kuhn 1989 ⁴⁵
		10.3mm dia., 5 mm cylinder	1.5–4.5†	10–500†	0.24 ± 0.09	—	Carter 1977 ³⁹
		5 mm dia./7.5 mm cylinder	5.96	103–1058†	0.46	—	Odgaard 1989 ⁴⁶
Monkey	Proximal tibia	7.5:7.5 mm cylinder	5.3 ± 2.9	445 ± 257	—	—	Linde 1989 ⁴⁷
	Vertebral body	Cylinders	—	165 ± 110	0.14 ± 0.06	—	Keaveny 1997 ⁴⁸
	Femoral head	5 mm dia./6 mm cylinder	23.1 ± 5.4	372 ± 54	—	—	Kasra 1994 ⁴¹
	Distal femur	5.5mm dia./8mm cylinder	8.5 ± 4.2	117 ± 61	—	—	Poumarat 1993 ⁴⁹
	Proximal tibia	15 mm cube, Ultrasonic method	—	648 ± 430	0.41 ± 0.16	—	Rho 1997 ⁵⁰
Cattle	Proximal humerus	Cylinders	—	1570 ± 628	0.71 ± 0.22	—	Keaveny 1997 ⁴⁸
	Vertebral body	6 mm dia./7.5 mm cylinder	7.1 ± 3.0	173 ± 97	0.45 ± 0.09	0.19 ± 0.06	Swartz 1991 ⁵¹
	Femoral head	5 mm cube	12 ± 5.8	435	—	—	Vabey 1987 ⁵²
	Distal femur	8 mm cube	7.1 ± 4.6	209 ± 140	0.44 ± 0.16	0.26 ± 0.08	Kuhn 1989 ⁴⁵
		4 mm dia./5 mm cylinder	13–28*	210–394*	0.69–0.98	0.40–0.56*	Kang 1998 ⁵³
Dog	Proximal tibia	4 mm dia./5 mm cylinder	5–24*	106–426*	0.41–0.83*	0.22–0.44*	Kang 1998 ⁵³
		12.5mm dia./10mm cylinder	—	301–850	—	—	Sumner 1994 ⁵⁴
		5 mm cube	—	344–1278	—	—	Sumner 1994 ⁵⁴

Goat	Humeral head	4 mm dia./5 mm cylinder	18 ± 6	350 ± 171	0.84 ± 0.17	0.43 ± 0.06	Kang 1998 ³³
	Distal humerus	6 mm dia./15 mm cylinder	13 ± 3	1490 ± 300	—	—	Kaneps 1997 ¹⁹
	Vertebral body	5 mm dia./8 mm cylinder	10.1 ± 2.6	530 ± 40	—	—	Acito 1994 ⁵⁵
	Femoral head	4 mm dia./5 mm cylinder	19.2 ± 6.9	502 ± 268	0.91 ± 0.04	0.48 ± 0.03	An (unpublished data)
	Distal femur	4 mm dia./5 mm cylinder	14.1–23.5*	399–429*	0.54–0.66*	0.32–0.40*	An (unpublished data)
	Proximal tibia	4 mm dia./5 mm cylinder	24.7–26.1*	532–566*	0.93–1.1*	0.50–0.56*	An (unpublished data)
Sheep	Humeral head	4 mm dia./5 mm cylinder	10.0 ± 1.0	247 ± 20	0.75 ± 0.03	0.36 ± 0.01	An (unpublished data)
	Femoral neck	8 mm dia./10 mm cylinder	3.2 ± 0.3	2.0 ± 0.2§	—	—	Geusens 1996 ⁵⁶
	Vertebral body	7 mm dia./9 mm cylinder	23.6 ± 4.4	—	—	—	Deloffre 1995 ⁵⁷
		7.5 mm dia./9 mm cylinder	22.3 ± 7.1	1510 ± 784	0.60 ± 0.16	0.37 ± 0.11	Mitton 1997 ⁵⁸
Pig	Vertebral body	7 mm dia./5 mm cylinder	27.5 ± 3.4	1080 ± 470	—	0.46 ± 0.04	Mosekilde 1987 ⁵⁹
Rabbit	Epiphyseal	Ground bone surfaces	35–81	—	—	—	An 1996 ²³
	long bones	Indentation test	—	—	—	—	—
Rat	Epiphyseal	Ground bone surfaces	38–71	—	—	—	An 1997 ⁶⁰
	long bones	Indentation test	—	—	—	—	—

† Range of values; *Range of average values from different parts; §Value is questionable (too low).

C. MEASUREMENT OF BONE DENSITIES AND MINERAL CONTENT

The density of a material is its mass per unit volume. The material density of cortical bone is the wet weight divided by the specimen volume. Cortical bone has a density of approximately 1.9 gm/cm³.^{80,81} The common ways to measure the volume of a cortical bone specimen include the use of a gravity bottle based on Archimedes' principle or directly measuring the dimensions of the specimen. The latter requires the specimen having a regular shape such as a cylinder.

For cancellous bone there are different material characteristics arising from the two phase structure (trabeculae and marrow).³⁹ Therefore, two mechanical properties are generally considered, the structural and material properties, which are based on their structural (apparent) density and material density, respectively. The measurement of structural (apparent) density (ρ_a) is achieved by weighing the cancellous structure without free water in its marrow cavities (wet weight, w_b) and dividing the wet weight by the structural volume (including both of trabeculae and marrow cavities):

$$\rho_a = w_b / (\pi d^2 h / 4) \quad (1)$$

where d and h represent diameter and height of a cylindrical specimen. Other specimen shapes, such as cubic, can be used, but they are technically more demanding and have more sharp corners than cylinders, which may cause bone materials to fracture from the specimen during defatting or marrow removal. An accurate method for bone volume is using a gravity bottle based on Archimedes' principle (before marrow removal). The compressive strength (σ in MPa) of cancellous bone is related to its apparent density (ρ in g/cm²) by a power law of the form:⁶

$$\sigma = 60\rho^2 \quad (2)$$

Similarly, the compressive modulus (E , in MPa) of cancellous bone is related to the apparent density (ρ , in g/cm²) by:

$$E = 2915\rho^2 \quad (3)$$

Selected reports on apparent densities of human and animal bones are listed in Table 3. The apparent density of cancellous bone ranges from 0.14 to 1.10 gm/cm³ (average: 0.62 gm/cm³, $n=16$).

Material density of cancellous bone is measured using the weight of bone material (only trabeculae) divided by the volume of only trabeculae, which is a little smaller than that of cortical bone, being 1.6–1.9 gm/cm³.⁸⁰ The principle is again that the marrow needs to be cleaned thoroughly before the measurements of weight and volume. Using a gravity bottle based on Archimedes' principle is the common way to measure both the weight and volume of the bone specimen. To make the measurement, the marrow has to be removed first and no air bubbles or water can be trapped inside the marrow cavities.

Many methods have been reported for removing bone marrow, including boiling in water with detergent, high pressure water jet, or chemical solvent. Depending on the size and shape of the specimen, an individualized combination of the above-mentioned methods is appropriate. In the authors' laboratory the following procedure has been used for small specimens (for example, 4 mm diam., 5 mm length cylinder): (1) Defatting in 50/50 acetone/ethanol mixture with agitation for 24 hours; (2) Removing marrow in low concentration bleach (1.0 to 1.5% sodium hypochlorite) with agitation for 12 hours; and (3) Removing marrow residues with a high pressure water jet (using a syringe).

For measuring bone mineral content, bone specimens are ignited in air in a 500°C furnace and the ash weighed. Instead of "ash weight" or "ash fraction," the authors prefer to use ash density, which is defined as ash weight per unit bone volume. It is suggested that the crucibles be dried at 500°C overnight, weighed, loaded with bone specimen, and heated at 500°C for 18 hours to remove

the organic phase. Then, the crucible containing the ash is weighed to determine the weight of ash. No predrying is needed for this method, while for ash fraction the specimen has to be dried at least for one week to get a base weight for calculating ash fraction. Selected reports on ash densities of human and animal bones are listed in Table 3. Less frequently used methods for determining bone mineral content include the use of decalcifying solution or measuring the radiographic density of whole bone or bone sections. The latter is more suitable for *in vivo* conditions.

Selected data of ash densities of human and animal bones are listed in Table 3, ranging from 0.19 to 0.50 gm/cm³ with an average of 0.37±0.10 gm/cm³ (n=13), which is about 60% of the value of apparent density. The latter is calculated from the 12 data sets containing both values of apparent density and ash density.

D. MECHANICAL TESTING AND DATA COLLECTION

1. General Consideration

Mechanical testing of materials involves the application of measurable loads to specimens of uniform dimensions. The applied stress is calculated by dividing the applied force by the area over which the force acts. Change in specimen dimension divided by the original specimen dimension defines strain. Dependent upon the direction in which the force is applied, the test may be tensile, compression, or bending. A simple way to record the data is a load-displacement curve from which the ultimate load, stiffness, and displacement are obtained. A stress-strain curve is not always plotted. Ultimate strength and elastic modulus (if applicable) are often calculated using the recorded loads, ultimate load and displacements, and dimensions of the specimen.

The mechanical test machine is operated in displacement control for most tests. The machine linear variable displacement transducer (LVDT) should be calibrated periodically using an extensometer. Loading is commonly conducted at a constant slow rate (1 mm/min is the rate used in the author's lab.). Load at the peak point of the load-displacement curve is taken as the ultimate load. A stiffness measure is obtained by measuring the slope of the linear portion of the curve (Figure 2). If the test machine is controlled with a linear displacement rate and if the specimen fixture is very rigid, the time base of the recorder can be converted to specimen deformation. An extensometer is recommended when a tensile test is conducted or for other tests when a complicated or less rigid specimen fixture is employed. The deformation measured by a built-in LVDT includes the deformation of the specimen and potential displacements within the specimen fixture or at the specimen-fixture interface. An extensometer attached to the specimen provides a direct measurement of specimen strain without the complications of machine or fixture deformation.

2. Artifacts of Testing Procedures

a. Machine Compliance

If several fixture parts are used in the testing assembly, a significant machine compliance can exist. A routine testing for machine compliance is recommended by testing the fixture column without specimen in place.^{23,58} If the machine and fixture deformation is found to be significant (more than 10% of the specimen value), it should be accounted for in the data analysis.²³ Because the testing gear should be the same for each specimen, only the mean machine compliance (or machine stiffness, S_m) is needed. The stiffness of the bone sample (S_b) is calculated using the following equations:

$$S_b = P/(d_{b+m} - d_m) \quad (4)$$

$$= P/(P/S_{b+m} - P/S_m) \quad (5)$$

$$= S_{b+m}S_m/(S_m - S_{b+m}) \quad (6)$$

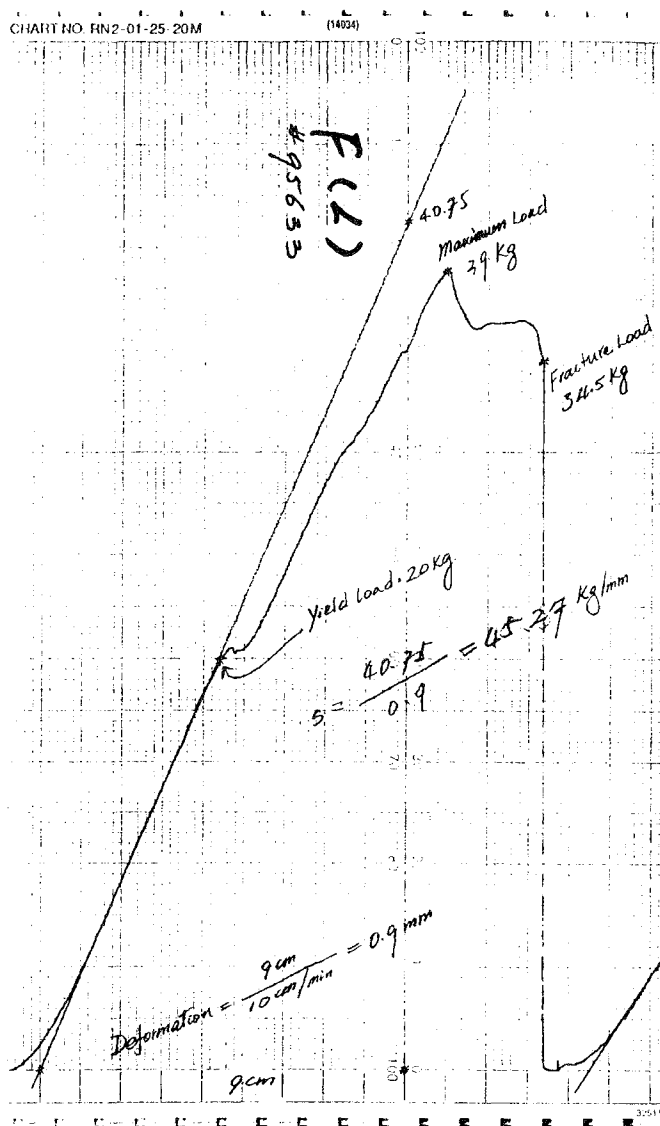


FIGURE 2. A typical load-displacement curve from a three point bending test on a rabbit femur.

where P is the load where deformation of the testing machine (d_m) or deformation of the machine plus bone specimen (d_{b+m}) are taken. S_{b+m} is the tested stiffness value (the stiffness of the machine plus bone specimen).

b. Specimen-Fixture Interface

When a tensile test is employed, the effect of specimen-fixture interface should be considered. Any loosening or low rigidity at the interface will lead to the underestimation of the specimen's true values. Therefore, a rigid connection between the specimen and the fixture is essential. Using a dumbbell-shaped specimen or PMMA end-coated specimen are two common strategies to achieve a good bonding between the specimen and fixture.^{28,82} An external extensometer should be used in this kind of situation to accurately measure the specimen deformation.

When a compression test is used, friction at the specimen and platens should be considered.⁸⁴ An uneven specimen surface causes a triaxial stress field, leading to overestimation of the specimen stiffness. This effect can be limited by using a more accurate procedure for specimen fabrication to achieve a parallel end surfaces. An overestimation of specimen stiffness can be also caused by the horizontal friction between the surfaces of the specimen and the platen. It is known that both the axial and lateral deformations of a specimen in between the upper and lower platen are larger at the ends of the specimen than the central part of the specimen (end phenomenon or end effect). Any restrictions to the lateral expansion, such as a rough platen surface, will cause an overestimation of the true specimen stiffness. Common methods for reducing this kind of friction include the use of grease at the interface and using low friction stainless steel platen surfaces (polished “mirror” surfaces).

For compression and tensile tests and the most of other mechanical testing procedures, pre-loading with a small load is useful for “tightening” the specimen-fixtured interface, to further limit the effect of the interface.

V. MECHANICAL TESTING TECHNIQUES

A. BENDING TEST

A bending test commonly is used for measuring mechanical properties of cortical bones.⁸⁵ Using bending tests, standard specimens cut from cortex or the whole intact bone can be used for testing. For the former, straight specimens (beams) with uniform cross-sectional shape and area are commonly prepared for testing. A three-point bending test is more often used than four-point bending, although theoretically it is not as sound as four-point bending. Elastic moduli determined from whole bone bending tests cannot be considered accurate because of the inconsistent cross-sectional shape and area, small length-to-diameter ratio (ideally, it should be 20:1, but for rabbit long bones only 6–8:1), and the inconsistent structures in the medullary canal (trabecular bone portion and marrow).^{28,80} However, in orthopaedic research, more and more whole bone bending tests have been reported using intact bone, which is likely because for most research projects, the relative differences between treated and control bones are of more concern than the actual values of the mechanical properties of the bone.

Bending tests have been used for testing long bones of mouse, rat, rabbit, cat, dog, and sheep. In such experiments, it is usually assumed that the cross-sectional area of the shaft of the bone is a hollow ellipse with walls of uniform thickness and the portion of the bone to be tested (in between the two supports) is assumed to be consistent in cross-sectional size and shape.

For a three-point bending test, the loading point is chosen at the mid shaft of the tubular bone. The surface facing the loading striker is often at the position of the smaller diameter simply because in this way the bone could be positioned steadily on the two supports. The specimen supports are commonly two steel rods, 4–6 mm in diameter with a span determined by the length of the bone. After the bone is positioned on the supports, the striker (4–6 mm diam. rod) is driven into the bone surface at a constant rate (1 mm/min. in authors’ lab.) until the bone fractures (Figure 3). The following equation is used to calculate the ultimate strength:

$$\sigma = PLa/8I \quad (7)$$

where P is the ultimate load, L is the distance between the supporting bars, a is the average value of the external diameters of the cross sections at the loading position of the bone being tested, and I is the area moment of inertia. The latter is calculated using the following equation:

$$I = \pi (a^3b - a'^3b')/64 \quad (8)$$

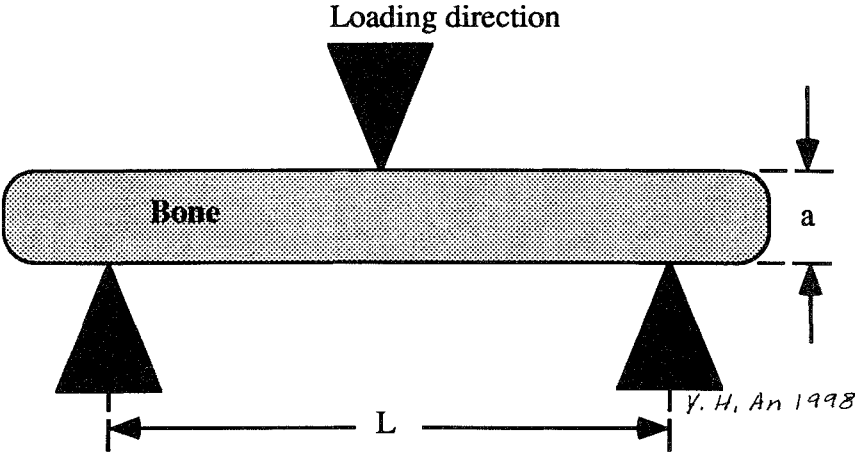


FIGURE 3. The setup of three-point bending test.

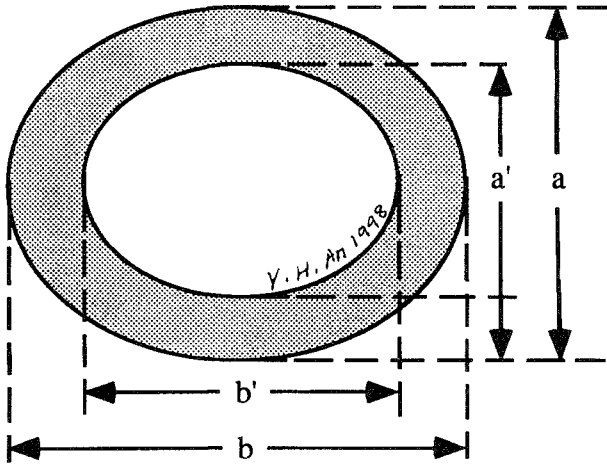


FIGURE 4. Illustration of the external and internal anteroposterior and side-to-side diameters for the cross-sections at the loading points of the bone (a, a', b, and b').

where a, a', b, and b' are the mean external and internal anteroposterior and side-to-side diameters for the cross-sections at the loading points of the bone (Figure 4). The external diameters (a and b) were measured before testing by use of a digital caliper. After testing, the pieces are glued together and cut transversely at the break point. The dimensions of the medullary canal (a' and b') are then measured. The following equation is used to calculate elastic modulus:

$$E = SL^3/48I \tag{9}$$

where S is the stiffness and L is the distance between the two supporting bars.

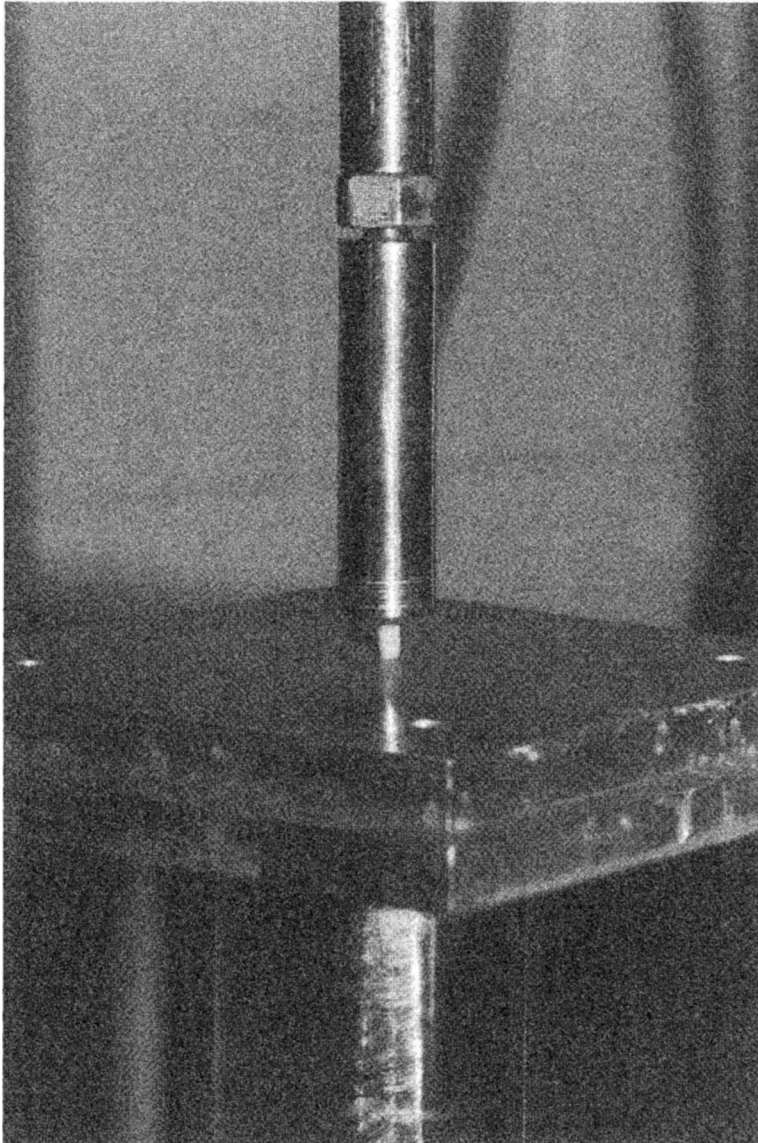


FIGURE 5. Photograph of compression test.

B. COMPRESSION AND TENSILE TEST

A compression test is commonly used for testing mechanical properties of cancellous and cortical bones.⁸³ Theoretically, a compression test is not as solid as a tensile test due to the edge effect and the axial inaccuracies.^{28,85} But, because of the easier sample preparation (commonly trephining), the compression test remains a popular and useful mechanical test.

For testing procedures, the testing machine should be operated under displacement control. The upper and lower platens should have polished stainless steel surfaces. Figure 5 shows a compression testing in action. Ultimate compression strength (σ) is calculated by the following equation if the bone specimen is cylindrical:

$$\sigma = 4 P/\pi d^2 \quad (10)$$

where P is the maximum load and d is the diameter of the bone cylinder. The elastic modulus by compression test (E) is calculated by:

$$E = SL/A \quad (11)$$

where S is the compression stiffness, L is the length of the bone cylinder and A is the end-face area of the specimen.

Keaveny et al.⁴⁸ compared the results by an accurate nondestructive method and the platens compression test and found a significant influence of specimen aspect ratio. A specimen with an aspect ratio of 2:1 (5 mm diam., 10 mm length) was the geometry which had the least difference between the two methods. They recommend that a 2:1 cylinder be used as a standard specimen in studies of uniaxial elastic modulus and strength of cancellous bone.⁸⁶

A tensile test is the standard engineering test for determining material properties. The specimen is machined into a beam with a smaller diameter in the central part of the beam (Figure 6). It needs a relatively complicated fixture with two universal joints above and below the specimen for specimen gripping and to line up the specimen. It has been successfully applied to both cortical^{28,42,87} and cancellous bone^{80,88} specimens. However, because of the difficulties of specimen fabrication, problems at the interface of specimen and grips of the machine, or simply because there is not enough volume of bone for making specimens, this test has not been utilized as commonly as compression testing for mechanical properties of bone, especially cancellous bones.

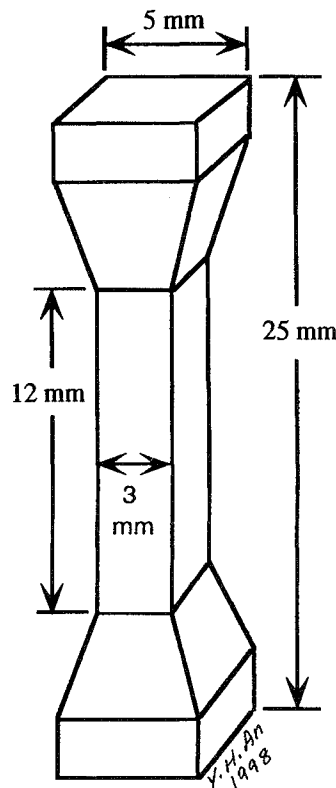


FIGURE 6. Illustration of a specimen fabricated with a gauge section reduced in size for tensile testing.

C. INDENTATION TEST

In the indentation test, an indenter is driven into a sectional surface of bone. Although the failure mechanisms are more complicated and less clear than the conventional compression test, it is useful for examining the mechanical properties of cancellous bones of different species.^{23,54,63,79,89–92} Because of the ease of specimen fabrication, the use of the indentation test has increased in recent years.²³ The test is simpler than the compression test which uses cubed or cylindrical samples. Only a flat surface of a sample is needed for indentation tests. The test is particularly useful for testing small bones. Recent reports describe the use of the indentation test for measurement of the mechanical properties of rabbit and rat cancellous bones.^{23,60} Indentation tests have also been used for testing the mechanical properties of fracture callus.⁹³

The selected bone specimen is cut and ground to a proscribed level or depth in the cancellous bone to create a surface for testing. After the first surface is created, a parallel cut is made to create a second surface to be used for setting the specimen against the specimen-holding platform. Instead of performing the second cut, the specimen can be potted in dental stone or plaster of Paris for positioning on the platform. The latter is especially suitable for small specimens. A cylindrical steel indenter with a flat end surface (2–5 mm diam.) is driven into the surface at a constant slow rate (1 mm/min.). Ultimate indentation strength is calculated using the following equation:

$$\sigma = 4 P/\pi d^2 \quad (12)$$

where P is the ultimate indentation load and d is the diameter of the indenter.

D. TORSIONAL TEST

Torsional tests are often used for testing mechanical properties of whole long bones such as the tibia or femur of dogs,^{64,94,95} rabbits,^{96,97} or rat.⁹⁸ The test is especially useful for testing larger bones. Bone ends can be embedded in plaster of Paris,⁹⁹ dental stone,⁹⁸ or epoxy resin⁹⁷ for mounting onto the testing machine. Commonly, maximum torque capacity (Nm), maximum angle of deformation (degree), torsional stiffness (N/m), torsional strength (MPa), shear modulus (MPa or N/m²) are calculated. Readers may refer to the books on this subject edited by Evans¹⁰⁰ and Hayes and Bouxsein.⁶

E. SCREW PULLOUT TEST

Screw pullout tests are used for testing the holding power of different screw designs. When one screw is used on different bone specimens,^{101,102} on bones treated with different preparation and storage methods,⁷⁷ or from different directions of one bone block,⁶³ variations of mechanical properties can be demonstrated. For the screw pullout test, a steel fixture is used, consisting of an upper screw gripping bar (connected to the load cell) and a lower specimen holding frame (Figure 7). It is always desirable to employ universal joints above and below the specimen for better specimen alignment. After the specimen is positioned on the fixture, the test machine is operated at a constant slow displacement rate (1 mm/min.) until the screw is pulled out. Ultimate strength (σ) is calculated using:

$$\sigma = P/\pi dh \quad (13)$$

where P is the ultimate pullout load (Newton), d (mm) is the major diameter of the screw, and h is the length of effective threads in the bone.

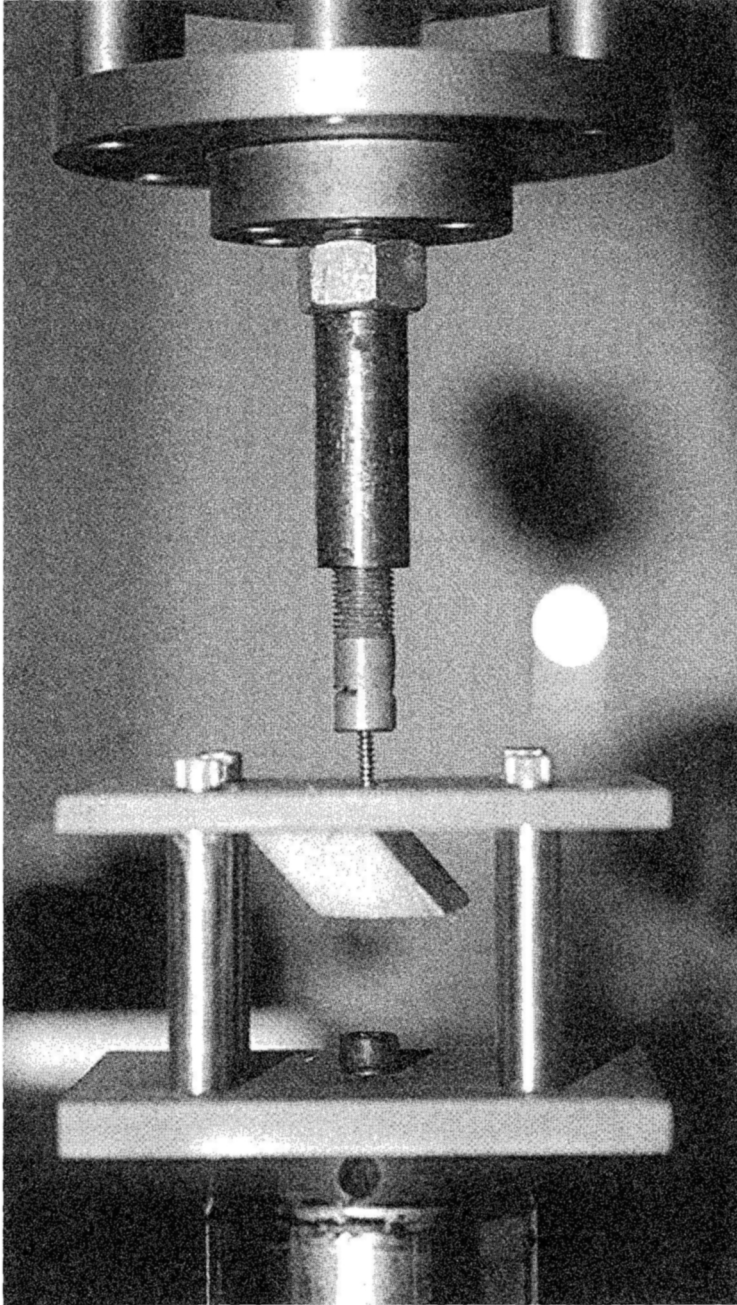


FIGURE 7. Photograph of screw pullout test.

F. STRAIN GAUGE

A strain gauge is a strain transducer made of etched foil grid patterns with a polymer backing. It is bonded to the bone surface, usually with cyanoacrylate adhesives, to measure bone strain. Single axis strain gauges measure strain in a particular direction while strain gauge rosettes (three gauges oriented at different directions mounted on a single polymer backing) allow the measurement of both magnitude and direction of principal strain.^{80,103,104} Strain gauges have been used in both

in vivo^{103,104} and *in vitro*¹⁰⁵ loading conditions. If the elastic modulus of the bone, location and orientation of strain gauges, and cross-sectional area and moment of inertia are known, then stresses, loads, and torque in the bone can be calculated from the measured strains.

G. ULTRASONIC METHODS

Ultrasonic techniques offer some advantages over mechanical testing for measuring elastic modulus of bone.⁸⁰ Specifically, the specimens can be smaller with less complicated shape (cylinder or cube) and several anisotropic properties can be tested using one (cubic) specimen.¹⁰⁶ The general relationship between elastic property (E) and the velocity of wave propagation (v) is as follows:⁸⁰

$$E = \rho v^2 \quad (14)$$

where ρ is the apparent density of the bone specimen. The exact form of Equation 14 depends on the mode (or direction) of propagation, the wavelength (for cortical bone: 2–10 MHz;¹⁰⁷ for cancellous bone: 50–100 kHz⁸⁸), and the cross-sectional dimensions of the material.^{80,106,107} Details of ultrasonic methods can be found in the papers by Ashman's group.^{80,82,88,106,107}

VI. TESTING OF BONE-IMPLANT INTERFACE

A. PUSHOUT AND PULLOUT TEST

The pushout test is the most popular test for evaluating the stiffness and strength of the bone-implant (cylindrical plug) interface.¹⁰⁸ Work done with a rabbit femoral condyle plug model is representative of the procedures of the pushout test.^{109–111} In this work, specimens to be tested (5.5 mm diam.) are prepared by sectioning the distal femurs containing the implant and hand grinding the bone sections to expose both ends of the implant. The specimen containing the implant is placed on the test assembly so that the implant is concentric with the hole in the specimen support (Figure 8). The diameter of the hole in the specimen-support of the pushout test assembly is 7.55 mm. The diameter of the loading rod should be smaller than the implant and large enough to provide uniform loading to the implant. The test machine is operated under displacement control mode with a ramp function and a displacement rate of 1.0 mm/min. Two important parameters should be calculated, interfacial stiffness and interfacial shear strength. The latter is calculated by dividing the ultimate load by the bone-implant interface area:

$$\sigma = P/A = P/\pi dh \quad (15)$$

where P is the maximum load applied to the implant, A is the nominal surface area of the implant, and d and h are the diameter and height of the cylindrical implant. Interfacial stiffness is taken as the slope of the linear portion of the load/deflection curve and has the units of MPa/mm.

Like the pushout test, the pullout test also measures the implant-bone interfacial stiffness and strength.¹¹² Theoretically, if testing two identical specimens (identical implants and bones) using pushout and pullout test (one test for one specimen), the results should be the same. The only difference in the tests is the mode of loading, pushing or pulling. It is more complicated to design a fixture for pullout tests than for pushout tests. Pullout tests require complicated fixtures with two universal joints for specimen gripping and alignment of the specimen. Pushout tests require only a push rod and a block of metal with a hole drilled in the center. However, the pullout test has some advantages over the pushout test. The accuracy of the pushout test depends on how well the two bone surfaces are prepared. The surfaces should be parallel to each other and perpendicular to the axial line of the implant. Another advantage of the pullout test is the improvement in specimen

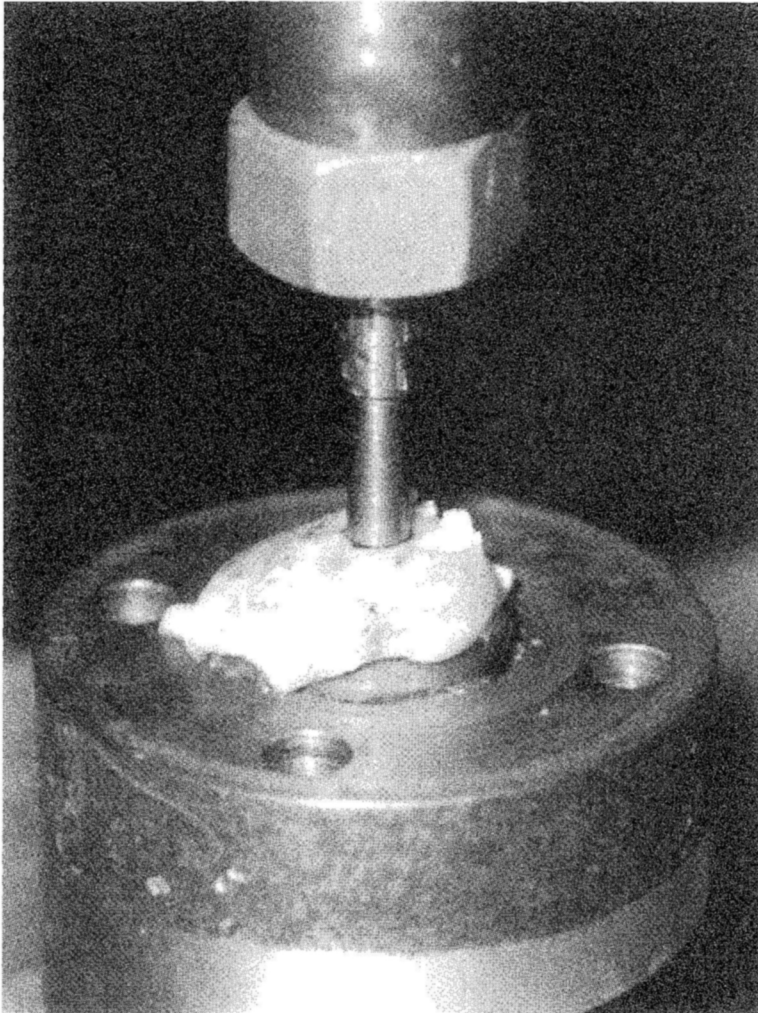


FIGURE 8. Photograph of pushout test.

alignment by the use of universal joints. For pullout tests, the implant-bone interfacial strength is calculated using Equation 15. Recently, a nondestructive pullout test has been proposed.¹¹³

The size of the bone is critical to the success of pullout/pushout tests. Rat bones are not appropriate for implantation using pushout or pullout testing because of their small size. Rabbit, canine or sheep femur or tibia are commonly used for metaphyseal or transcortical implant models. For cylindrical implants, the length/diameter ratio of the implant needs to be more than 1:1. Specimens with severe inflammatory changes or suspected infection should be excluded.

B. SCREW PULLOUT TEST

The holding power of different screw designs are tested using screw pullout tests.^{114–116} The key for designing a screw pullout test is that the bone specimen to be used should have uniform structural and mechanical properties so that differences measured are caused by different screw designs. Uniform materials, such as Dacro porous foam (a polyurethane foam formed by mixing a resin and an isocyanate),¹¹⁷ also can be used as a model system for screw pullout tests. The testing procedure is the same as that described in section V. Ultimate strength (σ) is calculated using

Equation 15. In the authors' laboratory, a pullout test has been used to test the holding power of a threaded cylindrical ligament anchor.^{118,119}

C. REMOVAL TORQUE

The bonding between bone and screw type implants can be evaluated by a removal torque method described by Carlsson et al.¹²⁰ The strength at the bone-implant interface is measured by using a torque wrench to determine the maximum torque necessary for manual removal of the implant.

D. OTHER TESTS

A variety of mechanical tests which combine and adapt aspects of the standard tests have been employed to study the properties of the bone-implant interface region. Of particular interest are two special tensile tests which have been reported for testing the bond strength at a bone-HA interface¹²¹ and a bone-cement interface.¹²²

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9 Mechanical Testing of Cartilage

Martine LaBerge

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I. INTRODUCTION

Due to a layered collagen network and the preferred orientation of the collagen fibers, articular cartilage is considered an inhomogeneous and anisotropic material. Both of these characteristics have been verified by tensile testing of cartilage sections obtained in different orientations.¹ This microscopic arrangement has been shown to influence strength and stiffness. Articular cartilage exhibits a nonlinear stress-strain behavior under large compressive strains² and also demonstrates a nonlinear stress-strain behavior in tension.³ When cartilage is placed under physiological compressive stresses, the modulus of the tissue increases with increasing strain, demonstrating an ability to limit excessively large strains in the tissue.⁴ The mechanical response of articular cartilage is time dependent, and exhibits phenomena such as stress relaxation, creep, and hysteresis. In addition, the material is sensitive to loading rate. Therefore, authors have used several mechanical tests to define its properties.

Commonly known experimental set-ups proposed for cartilage testing in compression include the confined and the unconfined compression test,⁵ and the indentation test. Confined compression tests consist of using an apparatus designed to restrict radial expansion of the specimen^{6,7} and allow uniaxial deformation.⁸ A typical experimental set-up for confined tests involves placing small cylindrical cartilage specimens, which are attached onto subchondral bone, in a confining chamber. The cartilage surface is in contact with a porous filter which allows free fluid flow as it is exuded from the tissue during compression. Tests are conducted either at room temperature or physiological temperatures. A small tare load (0.2 N) is applied to ensure that the specimen fills the confining chamber, and is in contact with the porous filter. Loads providing physiological stresses are then applied with a plunger contacting the subchondral surface and the resulting creep deformation recorded over time, until equilibrium is reached. This equilibrium condition can take 40–60 min.

as reported in the literature and defined by Kwan et al.⁹ Articular plugs must be excised for confined and unconfined compression tests.

Besides being the preferred method of characterization of the state of degeneration of articular cartilage in animal models, the biphasic indentation technique has also provided a means for the evaluation of Poisson's ratio,^{10,11} the shear modulus, and permeability of articular cartilage.^{12,13} An indentation test can be used to evaluate the response of excised cartilage or intact articular surfaces to compressive loading. It requires a testing apparatus that can control the applied load and monitor deformation.

The response of articular cartilage to compressive loading has been extensively studied through animal models in order to better understand mechanisms of degeneration due to aging,¹⁴ joint diseases, and following joint hemiarthroplasty.¹⁵ The mechanical testing of articular cartilage is either done *in vitro*, following the resection of the targeted tissue,¹⁵ or *in vivo* through arthrotomy,¹⁶ or arthroscopy.^{17,18} The major goal of these experiments is to determine the response of the tissue when subjected to the action of an applied load. An indentation of the tissue is normally performed to quantify the hardness and the viscoelastic behavior of cartilage in different environments. The coefficient of friction of the cartilage surface, an indication of the breakdown of its surface properties and its boundary lubrication are also measured experimentally. The response of articular cartilage to these modes of loading provides the basis for an assessment of the integrity or structure of the tissue, and of the potential failure of the cartilage, and depends on its structure at the time of testing. This chapter focuses on the use of indentation in the evaluation of cartilage properties, and presents an overview of different methodologies used by authors to characterize the properties of articular cartilage as often used in the design of orthopedic animal models.

II. INDENTATION TESTING OF ARTICULAR CARTILAGE

A. INDENTATION THEORY FOR COMPLIANT LAYERS

Any material can be indented, whether its behavior is elastic or viscoelastic as well as linear or nonlinear. However, many mathematical models of indentation assume linear elastic behavior to simplify the analysis and most of the quantitative indentation methods have been developed to characterize materials in the linear elastic region.¹⁹ The layer thickness affects the stresses in the material due to the influence of the substrate properties and interfacial boundary conditions. Many studies choose to relate the stresses in the layer to the stresses computed by an indentation of a half-space made of the same material which is given by Hertz theory for point contact.¹⁹ The contact mechanics of layers require a numerical solution of integral equations in order to account for the additional length variable of the layer thickness and the boundary condition at the base of the layer. Such solutions are not as easy to apply as the standard Hertz equations.

For linear-elastic materials, data from an indenter test typically include the depth of penetration and the applied load. The data can then be used to compute the stresses if the material properties are known. Also, if the area of contact is experimentally measured, the local material properties can be determined. A general empirical equation (Eq.1) for a linear-elastic (rubber) thin layer indented by a rigid sphere and bonded on a rigid substrate was proposed by Finkin.²⁰

$$E = \frac{9PR}{16H^3} \left[\left(\frac{Rd}{H^2} \right)^{0.5} + 0.252 \left(\frac{Rd}{H^2} \right) + 0.1588 \left(\frac{Rd}{H^2} \right)^{1.5} + 0.2245 \left(\frac{Rd}{H^2} \right)^{2.0} + 0.3069 \left(\frac{Rd}{H^2} \right)^{2.5} + 0.298 \left(\frac{Rd}{H^2} \right)^{3.0} \right]^{-3.0} \quad (1)$$

where E is the elastic modulus, R the radius of the indenter, H the depth of penetration of the indenter, P the load, and d the thickness of the specimen. This model derives explicit equations for Young's modulus in terms of either penetration of the indenter or contact radius for a rubber layer of thickness greater than the radius of contact.

B. SPECIFICATIONS OF ARTICULAR CARTILAGE INDENTATION

An indentation test is a sensitive way to quantify the viscoelastic response of cartilage by displaying both elastic and time-dependent properties under a compressive load.⁴ While the tissue remains intact on the joint surface, a nominal static compressive load is applied with an indenter and the deformation of the cartilage is monitored. Upon unloading, an instantaneous recovery of the cartilage deformation is followed by a time-dependent recovery.²¹ The monitored deformation of the test cartilage during loading is plotted against time and compared to that of normal cartilage, a contralateral joint, or different locations on the same joint surface. Modifications of the mechanical properties of articular cartilage induced by aging and pathological degeneration processes were investigated with indentation tests. Hirsch,²² Sokoloff,²³ and Kempson et al.²⁴ determined that the degenerated cartilage on subchondral bone was more compliant than normal cartilage when loaded compressively with an indenter.

Hayes et al.²⁵ used a numerical analysis to derive a set of equations and constants that can be used to determine the Young's modulus and the Poisson's ratio of cartilage from indentation test measurements. In this case, the cartilage was modeled as a layer of linear-elastic material and an analysis was applied to the two extremes of an indentation curve: the initial response (immediately after loading) and the final deformation. Hayes et al.²⁵ derived two solutions for plane-ended and spherically ended indenters and their dependence on the contact radius, Poisson's ratio, and surface stresses were studied. Even though this model was not experimentally validated, researchers have used these equations to obtain material properties for articular cartilage. This model assumed the material to be homogeneous.

Even though an indentation test appears by definition to be a simple approach to quantify cartilage degeneration or evaluate its mechanical properties, there are many difficulties inherent to indentation measurements consequently leading to unreproducible and inaccurate data. Therefore, certain points must be addressed when designing an indentation test. These include: (1) design or selection of an apparatus that can accurately measure cartilage deformation accounting for the geometry of the indenter, (2) the selection of loading conditions such as the rate of loading and the load applied, (3) the environmental conditions during testing, and (4) the information to be extracted from the curves. The following conditions should be respected during indentation testing:

- The load must be applied perpendicularly to the cartilage surface in order to obtain an axially symmetric loading on the tissue. In this respect, tare loads used to facilitate the alignment of the indenter deform the cartilage surface and should be carefully monitored since they will modify the resulting indentation even though very small.
- Given that the cartilage surface is curved, alignment of a plane-ended indenter is very difficult, and leads to the formation of high stress concentrations resulting in cartilage damage. The use of spherically-ended indenters allows for a better alignment and for a constant contact with the surface and does not cause stress concentrations.
- The load applied on the surface should remain constant during the indentation to minimize variation in indentation depth due to load application. Load application techniques should be selected to minimize excessive loads due to inertia.²⁶ Swann and Seedhom²⁶ have recommended the use of linear bearings, and Athanasiou et al.,¹³ air bearings. The rate of loading must also be controlled since cartilage is a viscoelastic material. The deformation therefore is dependent on its loading rate, so it will appear stiffer loaded at high strain rates than at low strain rates.
- A common limit of most commercial testing equipment is the precision of the equipment. As an example, a strain of 10% measured with a precision of 1% in a sample measuring 10 mm requires a displacement precision of ± 0.01 mm, a common limit in most commercial testing equipment. Cartilage samples are normally in the range of 1 mm thick

requiring displacements of $\pm 1 \mu\text{m}$. Aspden et al.⁴ proposed a computer-controlled mechanical testing machine for small samples of biological viscoelastic materials as articular cartilage. This apparatus allows displacement in steps of about $1 \mu\text{m}$ in a predetermined time.

- The simulation of physiological environmental conditions will allow for a closer representation of the behavior of the cartilage in its natural environment. Such systems should allow humidity and temperature to be controlled to simulate physiological conditions.^{15,16} Elmore et al.²⁷ experimentally demonstrated that the deformation and recovery of cartilage depends on the fluid movement between the interstitial liquid and the environment. The accuracy of an indentation test is based on the presence of a liquid environment allowing for a recovery of the deformation after load removal. According to Tkaczuk et al.,^{28,29} a change in properties might occur by alterations of the hydrochemical properties of the tissue after resection of the tissue. Therefore, immersion of the resected cartilage in physiological saline is the common practice during indentation tests.¹⁵
- The deformation of articular cartilage under compressive loads is influenced by its thickness. As discussed earlier, the cartilage lying on subchondral bone can be represented by an elastic layer connected rigidly to a rigid base. Therefore, the measurement of the cartilage thickness is an intrinsic part of an indentation protocol. Cartilage thickness can be measured on resected plugs.^{9,30,31} However, sectioning results in changes in cartilage thickness as a result of dehydration or swelling depending if the tissue is in contact with air or with physiological saline. Rushfeldt and Mann³² suggested a non-destructive method to measure the thickness of articular cartilage *in vitro* where a detailed thickness distribution of the articular cartilage in the human hip joint was obtained using an ultrasonic transducer. The cartilage thickness was measured with the pulse-echo "A-scan" ultrasonic technique, in which the ultrasonic transducer produced a pressure pulse that traveled through the immersion medium as a longitudinal wave. As the wave passes through interfaces between media of different acoustic impedances (such as cartilage surface, calcified cartilage, and bone) reflections return to the transducer and generate electrical signal in the transducer proportional to intensity. However, other authors have reported several difficulties when using ultrasound to measure cartilage thickness.⁴⁰ The use of a needle attached to a micrometer and inserted in the tissue was also proposed as a measurement technique.³³

C. LUBRICATION OF ARTICULAR CARTILAGE AND FRICTION MEASUREMENT

The synovial joint is a highly efficient bearing with two major types of lubrication regimes: fluid lubrication and boundary lubrication. In conditions of low speed and high load, fluid lubrication mechanism is assisted by boundary lubrication achieved in a layer of molecules attached to the surface of cartilage also known as surfactant.³⁴ Low coefficients of friction (μ) have been measured in healthy joints (from cadavers) with values ranging from 0.0044 to 0.042, suggesting that fluid film would be the primary mode of lubrication.⁴⁰ Yet, the operating conditions seem unfavorable to fluid film lubrication due to impact loads and oscillating motion, conditions better supportive of boundary lubrication as observed by Barnett and Cobbold.³⁶ Additionally, the coefficient of friction decreases progressively as the load on the joint increases.³⁷ This situation is a classic indication of boundary lubrication. The impairment in the cartilage surfactant layer or its boundary lubrication properties would be an indication of a degeneration of the tissue.³⁸ Experimental set ups used to assess the tribological properties of cartilage tissue have mainly associated with normal cartilage. Animal or human articular surfaces are articulated against a mating cartilage surface or an artificial material such as metal or glass. Oscillatory (pin-on-disc system) or reciprocating motion is used. The tissue is normally kept under lubricated conditions in an environmental chamber simulating

physiological temperature and atmosphere. Lubricants such as physiological saline, synovial fluid obtained from the joint at resection, and culture media typically are used. A compressive load is applied approximating physiological stresses, and the friction test is conducted at 1 Hz or other frequencies of physiological relevance. Test duration or number of cycles should be selected to provide the best assessment of the frictional behavior of the articular surface without leading to excessive post-mortem degeneration of the tissue.³⁹ Static and kinetic coefficients of friction are measured using different means, load cells and force transducers being commonly used.⁴⁰

D. USE OF INDENTATION AND FRICTION MEASUREMENTS IN AN ORTHOPAEDIC ANIMAL MODEL

A study conducted by Hall⁴⁰ involved the use of indentation and frictional measurements to characterize the long term effect of an injectable non-steroid anti-inflammatory drug (NSAID), ketorolac tromethamine (Syntex Laboratories, Palo Alto, CA), used intra-articularly on the properties of lapine knee cartilage. In this model, the mechanical properties and tribological properties, focusing on the boundary lubrication, of articular cartilage were compared to its microstructure.

A total of six one month old NZW female rabbits were used for the study. Both knees were shaved and prepped with an alternating series of absolute alcohol and Betadine solution. The right knee was injected with a 0.5 cc solution of 30mg/ml NSAID and the left knee with 0.5 ml of sterile Ringer's solution. The rabbits were housed for 12 months, exercised weekly, and then euthanized in a CO₂ chamber following anesthesia and injections of xylazine (0.15 ml/kg body weight) and Ketaset (0.2 ml/kg body weight), according to the standards of the American Veterinary Medicine Association. Two additional 12 month old female rabbits were euthanized using the protocol described previously and used as controls. The guidelines of the PHS guide for care and use of laboratory animals were followed during the study. The knees were then resected. Each tibio-femoral joint was taken undisturbed and placed in a bag of sterile saline. The joints were then cleaned and all extraneous tissue removed, only leaving the bone, cartilage, and a few ligament attachments. The condyles were separated along the patellar groove. The testing procedures were conducted on fresh condyles.

An indentation test was conducted in order to evaluate the effect of NSAID treatment on the compressive behavior of articular cartilage. In this study, a desktop tensile testing machine was used for indentation testing (Vitrodyne V1000, Chatillon, NC). This benchtop testing system is designed to measure the mechanical properties of materials on a small scale as compared to standard testing specimens. This equipment consists of a precision linear actuator located on a testing stand, a control and interface module, interchangeable load cell, and custom computer software for machine control and data analysis. A precision linear actuator is mounted on a high-strength testing frame and used to convert rotary motion of a screw to slow linear motion of the main loading axis. The actuator is coupled with an optical position encoder to assure a displacement resolution of 1.0 μm for a total travel distance of 7.5 cm. The system can be used with nine interchangeable load cells, with maximum capacities from 30 grams to 10 kg with a force measuring accuracy of $\pm 0.5\%$ (2 to 100% of load cell capacity) and a load cell resolution of 0.05% of load cell maximum capacity. Testing can be conducted with a strain rate on the order of 10 $\mu\text{m}/\text{sec}$ up to 200 mm/sec. Standard grips for test specimens can support testing in tension, compression, and bending. Specimens can be submersed in fluids or mounted in an environmental chamber. This system is fully computer controlled. The software specifically designed for this system features real-time graphic display of test results and supports standard tests such as load-displacement, stress-strain, stress relaxation, and continuous dynamic cycling at fixed or scanning frequency. Testing was performed in physiological saline at room temperature. Each test was completed on six different condyles for both the NSAID and saline injected knees, as well as four condyles for the control group.

An acrylic specimen holder mounted on a bearing system was designed to allow rotation of the condyle fixed with polymethylmethacrylate (Simplex, Howmedica) to any angle that would

allow a perfect alignment of the indenter on the surface and to locking into place for testing. One indentation was performed on the selected condyles using a highly polished titanium alloy (Ti-6Al-4V) spherically ended cylindrical indenter with a radius of 1.5 mm. Load-displacement curves were obtained for a maximum indentation depth of 500 μm at a rate of 10 $\mu\text{m}/\text{sec}$. The system was activated and the indenter was positioned perpendicularly to the cartilage surface with a maximum preload of 0.5 g. After testing, the cartilage thickness was measured and the specimens were fixed in 10% buffered formalin solution for soft tissue histological preparation. The cartilage thickness was measured using techniques proposed by others.³³

A pin-on-disc friction apparatus was specifically designed to assess the effect of intra-articular injection on the friction coefficient of the cartilage of remaining femoral condyles (Figure 1). This system allows for a normal force to be exerted along the vertical axis providing a point contact geometry between the specimen and bearing surface. The support does not hinder any measurement because the lubricant and bearings in the actuator housing allow for near frictionless movement and allow normal force to be applied directly to the specimen. The shoulder joint which is the hinge upon which the specimen rotates is also fitted with bearings and coated with lubricant, in order to reduce any frictional or external forces. A load cell (1000G, No. 360817, Omega Inc.) is fastened securely on the stationary plate in line with the axis of the actuator in order to provide a horizontal force, which can be regarded as the resistance of movement of the specimen, thus the frictional force. The voltage of the load cell is transmitted to a data acquisition package (Labview 3, National Instruments, AZ) and converted into gram force. A magnetically geared motor (Model 4Z728A, Dayton Electric Inc., IL) was used and tests were conducted at a frequency of 1 Hz. The frictional force was monitored for a total travel distance of 50 m. Highly cleaned flat glass plates (mean roughness average [Ra] = 0.72 ± 0.09 nm, Topo-3D, Wyko Corp., 20X magnification head) were used as counterpart surfaces. A new cleaned plate was used for each test. Friction tests were conducted in a Plexiglass chamber at room temperature in a saturated nitrogen environment using normal saline as a lubricant to prevent cartilage dehydration. Tests were conducted at an entraining velocity of 30 mm/sec with a load of 600 g providing an average contact stress of 2.75 MPa. The calibration curve for the load cell was used to determine the frictional resistance based on the voltage output recorded during each test. The use of these values enabled the calculation of the coefficient of friction for each treatment. The signals were filtered through a low-pass resistor/capacitor filter in order to minimize the amount of low frequency noise.

A structural analysis was conducted to characterize the cellularity and the structural integrity of the test specimens. Histological sections of femoral condyles were stained with a safranin-O stain and hematoxylin-eosin stain. The image analysis of the histological sections was conducted by mounting a video camera (Sony, AVC-D7), powered by a camera adapter (Sony, MA-D2, Japan) to a large Zeiss Universal Research Microscope (Oberkochen, Germany) to capture the image observed under the microscope. The captured image was transferred to a monitor (Panasonic, WV-5410) and sent to a computer (Macintosh Quadra 900) to be digitized and saved. The actual cellularity determination was done by using the threshold mechanism in the imaging software package (Adobe Photoshop and NIH Image). The grayscale image could be thresholded into a density pattern and the nucleated cells would be differentiated from the rest of the tissues. The software could then tally the number of threshold areas which gives the number of cells per image. This analysis was conducted for 20 sections on all samples.

Results have shown that the control condyles required an average of 249.6 ± 19.3 gm, an average of 81.3 ± 23.1 gm for the saline group, and 123.4 ± 54.3 gm for the NSAID treated group for 20% deformation. The NSAID treated specimens were statistically more compliant than the control specimens but not different from the saline treated surfaces (ANOVA, 95% confidence interval).

For friction data analysis, the static coefficient of friction was chosen to be an average value of the first measurement for each experiment in one group. The kinetic coefficient of friction was defined as the average value of the remaining linear points in the data set and was evaluated at

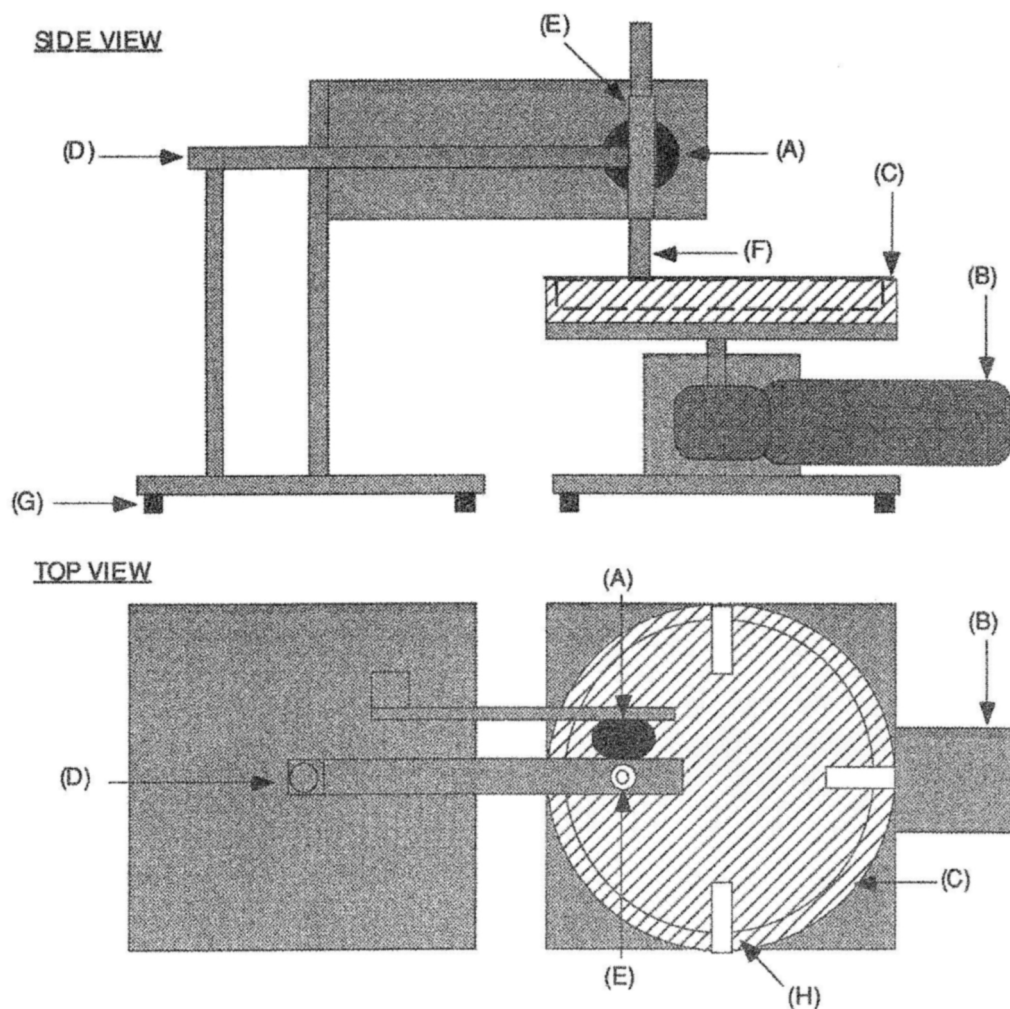


FIGURE 1. Schematic representation of the pin-on-disc friction apparatus specifically designed for articular cartilage testing: (A) 1000G load cell, (B) magnetic geared motor, (C) acrylic bowl and plate holder, (D) lubricated shoulder joint, (E) lubricated casing for actuator, (F) actuator and specimen holder (Dayton Electric Co.), (G) rubber feet, and (H) acrylic clamps for counterpart plate.

10 m sliding distance intervals. The statistical analysis (ANOVA, 95% confidence interval) revealed no significant differences between the test specimens, saline and NSAID, and the control specimens for the static coefficient of friction. However, during the kinetic phase the NSAID had a much higher average than the control and saline. This trend was followed throughout the linear region of the trial. A significant difference was determined for all of these cases. The difference remained significant as a function of time, and the NSAID samples remained higher than both the control and the saline samples. No statistical difference was observed between the saline and control specimens.

A statistically significant hypercellularity was observed for the NSAID specimens compared to the other groups. No statistical difference was observed for cellularity between the saline treated and control condyles. Overall, saline and NSAID groups had only 80% of the area positively stained

for PGs as compared to controls. However, the superficial zone of NSAID specimens was abnormally positively stained for PGs. A decrease in stiffness was also correlated to a decrease in overall positive staining for PGs.

In conclusion, the properties of articular cartilage were successfully investigated using indentation and friction analysis. Results have shown that the intra-articular injection of ketorolac-tromethamine affects the structural properties of articular cartilage, associated with an alteration in the positive staining for proteoglycans in the matrix and cellularity, as well as its mechanical and tribological properties as compared to normal cartilage. The characterization of the tribological properties, mainly friction coefficient, allows for the evaluation of the response of articular cartilage to a specific experimental treatment.

E. *IN VIVO* INDENTATION TESTING

The indentation test remains a useful tool for assessing the compressive stiffness of cartilage, especially in survey work where the objective is to compare between cartilage stiffness rather than to obtain absolute values of intrinsic mechanical properties.²⁶ Indentation tests can be classified into two types: *in vitro* or *in vivo* indentation testing. Most of the mechanical apparatus and systems proposed by authors for the indentation of articular cartilage involve *in vitro* testing following the resection of the specimens as discussed previously. During *in vitro* testing, the post-mortem degeneration of cartilage should be minimized and mechanical tests performed in a few hours to resection or preserve in conditions that would not affect its properties.^{41,42} Since indentation tests normally are performed on test and control specimens in orthopedic animal models, the preservation technique selected should be used for both groups of specimens.

Tkaczuk et al.²⁸ and Tkaczuk²⁹ pointed out the importance of performing mechanical indentation testing of cartilage in its physiological environment *in vivo* and proposed a cartilage elastometer for use on articular joints during orthopedic procedures. The apparatus essentially consisted of a device for attaching the elastometer to the bone, and force and displacement transducers. This procedure involved an arthrotomy of the joint to fix the elastometer to the bone. The deformation of cartilage as a response to compressive loading required breaking through the cartilage surface for measurement and, therefore, can be considered a destructive method. LaBerge et al.¹⁶ developed a non-destructive invasive method for *in situ* indentation testing to quantify cartilage deformation following an arthrotomy. The degeneration of articular cartilage in a canine closed chondromalacia patellae model was monitored quantitatively as a function of time. The portable autoclavable indentation system consisted of a load application device, a displacement measuring device, and a positioning device. The system subtracted out the deformation of the surrounding soft tissue and provided a more realistic measurement of the cartilage deformation. Depths of indentation and hardness of cartilage have been measured with an accuracy of 0.005 mm and a repeatability of 0.6%. A data acquisition system allowed to monitor cartilage deformation under a spring loaded hemispherically ended indenter.

A non-destructive approach was used by Dashefsky¹⁷ to measure the chondromalacia of the medial patellar facet. Under arthroscopic control, a microminiature pressure transducer was used to measure the resistance of cartilage to a predetermined amount of deformation. This technique was used in a clinical trial and intended to provide standard data to measure patellar softening. The advantage of this innovative technique is the use of arthroscopy. However, the testing technique proposed by Dashefsky¹⁷ did not provide a method to measure the angle between the probe and the cartilage, or the positioning of the device. Also, the use of a pressure transducer suffers intrinsic limitations. The required force (input) to apply a pressure should be kept constant and monitored, and the pressure (output) should be measured as a function of time since the cartilage is a viscoelastic material. The non-monitoring of these two factors can jeopardize the data interpretation and comparison. An indenter for use during arthroscopic procedures that allows testing of cartilage stiffness under minimally invasive procedures was also proposed by Lyrra et al.¹⁸ This instrument

imposes a constant deformation on the cartilage and the maximal indentation force by which the cartilage resists the deformation, is used as a measure for cartilage thickness. This instrument was also tested on cadaver knees and on elastomeric sheets and provided reproducible data.

III. CONCLUSION

Despite the fact that articular cartilage is a very complex material in terms of structure and mechanics, several mechanical tests can be performed on articular cartilage to assess its integrity or evaluate the effects of orthopedic procedures or implant materials on its behavior. Indentation testing of cartilage has been demonstrated by several authors as an effective method to observe the response of cartilage to compressive loading. The design of an indentation protocol should allow for reproducibility and accuracy, and should take into consideration the time-dependent response of the tissue, its biphasic nature, and its geometry (thickness and curvature).

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10 Mechanical Testing of Ligaments and Tendons

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I. INTRODUCTION

Ligaments and tendons are soft connective tissue structures which work in conjunction with each other to stabilize a single synovial joint. They are composed primarily of water and parallel oriented collagen fibers. The collagen is primarily Type I (approximately 70% dry weight), a small amount (3–10%) of Type III, and minute amounts of Types V, X, XII and XIV. Other non-collagenous proteins are present as well, including proteoglycans and elastin.

The longitudinal arrangement and crimped nature of the collagen fibrils serve to both guide joint motion and provide restraint at extremes of motion; in other words, at low loads the crimp pattern straightens easily so that joint motion is guided. At higher loads, the fibrils are straightened and loaded in tension, thus restraining excessive joint motion.

In this chapter, various methods to determine the biomechanical properties of ligaments and tendons will be discussed. With respect to animal models, the effects of experimental, biological

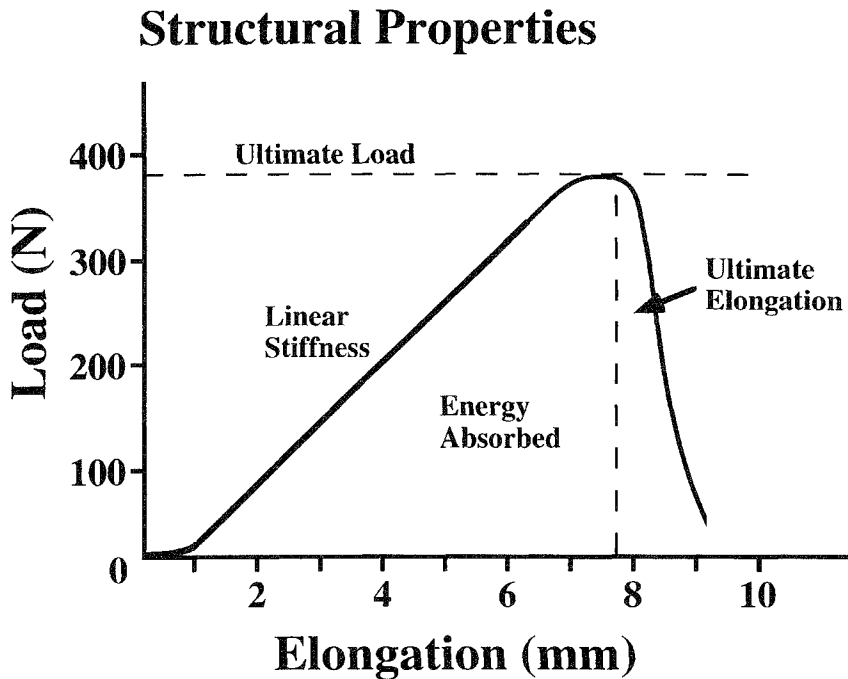


FIGURE 1. Typical load-elongation curve of a bone-ligament-bone complex.

and environmental factors on these properties will be reviewed. The flexor tendons of the hand and foot and the MCL of the knee are the two primary examples that will be our focus in this chapter because they are among the most studied; however, there is considerable information available on other ligaments and tendons as well, from shoulder to spinal ligaments to patellar and extensor tendons.

II. BIOMECHANICAL PROPERTIES OF LIGAMENTS AND TENDONS

Mechanical testing is used to elucidate the inherent mechanical properties of a material. Since ligaments and tendons are primarily loaded in tension, the result of a tensile test, the load-elongation curve is used to obtain ligament and tendon uniaxial properties. In the following discussion, the testing of ligaments will be used as examples. From the data curves, *structural* properties of a bone ligament bone complex and *mechanical* properties of the tissue substance can be calculated.

When we elongate a bone-ligament-bone complex in the materials testing machine, we simultaneously measure the load in this structure corresponding to that elongation from its resting length. Plotting the elongation as the independent variable and load as the dependent variable, a non-linear load-elongation curve is obtained (Figure 1). From this curve, important properties related to the entire bone-ligament-bone complex as a whole can be inferred. These properties include the linear stiffness (in Newtons/mm), the ultimate load (in Newtons), the ultimate deformation (in mm), and the energy absorbed at failure (in Newtons-mm).

The *mechanical* properties of the ligament substance can also be determined from the same uniaxial tensile test by normalizing both the load and elongation. When the load is normalized by the cross-sectional area to account for different widths and thicknesses, a quantity known as the

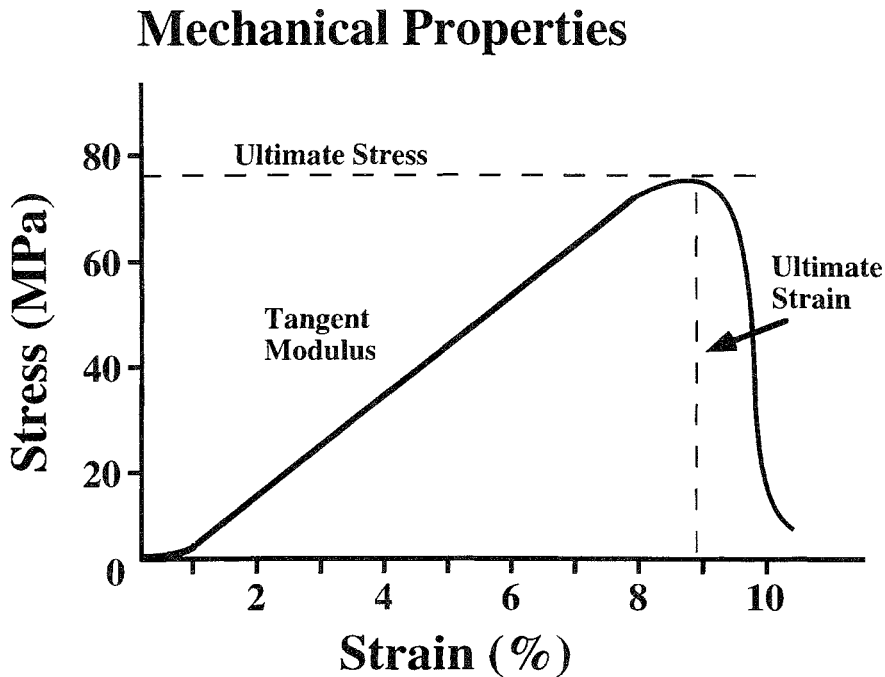


FIGURE 2. Typical stress-strain curve describing the mechanical properties of the ligament substance.

stress in the tissue is defined. Similarly, when the elongation of the specimen is normalized by the resting length, or “gauge length” of the specimen, a quantity known as the strain in the tissue is defined. Plotting the strain as the independent variable and the stress as the dependent variable, a non-linear stress-strain curve can be obtained for the ligament substance as shown in Figure 2. Between the range of strain 1–8% in this figure, the stress produced in the tissue is linearly proportional to the strain and the slope of the curve in this region is defined as the tangent (or Young’s) modulus of the material. A ligament (or tendon) with more collagen cross linking or stouter collagen fibrils will be more difficult to elongate or strain and will show a *higher* Young’s modulus.

The reason that the linear portion of the stress-strain curve is used to define Young’s modulus is that any elongation or strain is reversible. Unloading the specimen will cause the specimen to contract back to near its initial length. There is no permanent change in the material. At around 8% strain, the stress/strain curve shows the stress/strain curve becoming nonlinear. If stressed to this high degree, the tissue will not return to its previous resting length after relaxing the load. This is known as the yield strength of the tissue. When the stress in a ligament exceeds this point, the effectiveness of the ligament is reduced because the ligament now has to be elongated more before it will start to resist the elongation. The point on the stress-strain curve where the material actually fails is known as the tensile strength of the material and represents the maximum stress that the material can sustain prior to failure.

Much work has been performed in the determination of these biomechanical properties. However, the task of measuring many of these properties is challenging. There are many experimental and biological factors that can affect the outcome. In the following sections, these factors will be discussed in detail.

III. EXPERIMENTAL FACTORS

A. STRESS MEASUREMENTS: THE DETERMINATION OF CROSS-SECTIONAL AREAS

The cross sectional areas of ligaments and tendons are very difficult to measure because they are irregular in shape and also soft and deformable. The literature is divided on the approach to use, consisting of contact and non-contact methods. The contact methods include such methodologies as using calipers to measure the width and thickness of the specimen,¹ forming molds of the specimen,^{2,3} and the area micrometer system. The area micrometer forces a ligament into a slot of known width. The micrometer mounted indenter is used to compress the specimen into a rectangular shape to measure its height. As with other contact methods, the measurements are dependent on the amount of pressure applied to the specimen.⁴⁻⁶ Due to the fact that these methods alter the shape and cross section of the soft tissue during measurement, other investigators have preferred newer methods based on using non-contact technology. Examples of this technology include the shadow amplitude method⁵ and the profile method.^{4,7,8}

In our research center, a non-contact method using a laser micrometer system has been developed.^{9,10} Utilizing a collimated laser beam field and a background screen detector, we can accurately map the surface profiles of a ligament or tendon. The data is reconstructed such that an accurate assessment of specimen cross sectional shape and area can be obtained. A study comparing this technology to digital caliper and area micrometer measurements determined that while the cross-sectional area measurements (of the relatively flat medial collateral ligament (MCL) using an area micrometer were 21% less than those using the laser micrometer while the digital calipers overestimated the cross sectional area by 2.3%.⁹ Having proven to be accurate and reproducible, the laser micrometer system presents an excellent alternative to contact methods of cross-sectional area measurement. The one limitation of this system is its inability to measure the concavities present on the ligament surface such as those in the anterior cruciate ligament (ACL). While the number of ligaments exhibiting this geometry is minimal, additional methodologies utilizing laser reflectance transducers have also been developed.¹¹ The laser reflectance transducer uses an emitter to project a 1mm laser beam onto the surface of the specimen. A receiver collects the laser light reflected off of the specimen while the whole system is rotated 360 degrees around the specimen (Figure 3). From this data, accurate cross-sectional shape can be determined so that it can be integrated to determine the cross-sectional area. Since this system takes into account concavities present in the specimen, it does provide a realistic reconstruction of cross-sectional shape.

B. DETERMINATION OF STRAIN

In order to effectively measure the strain present in soft tissues during a uniaxial tensile test, accurate measurements of the initial length of the specimen and the elongation at any point during the test must be made. Many different methodologies have been used to measure this elongation.^{6,12-17} Literature use of percent elongation based on the clamp to clamp distance involved contributions of not only the ligament itself, but also its insertions. To measure the strain in the tissue, however, measurement of the strain in the ligament substance must be made. The technology available to measure tissue strain can again be divided into contact and non-contact designs.

Contact methods of measuring strain in ligament or tendon tissue include the use of strain gauges and Hall effect transducers.¹² Liquid mercury strain gauges were used by some researchers to measure the percent elongation in knee ligaments.¹⁸ Both of these contact methodologies again involved direct interference with the tissue during testing, which may introduce errors into the measurements.

In our research center and others, non-contact methods of measuring strain have been used. Due to the fact that continuous measurements of tissue length must be made, two systems have enjoyed widespread use. The first is the video dimension analyzer (VDA) system.^{19,20} In the use of this system, reference lines are marked perpendicular to the loading axis with Verhoff's elastin

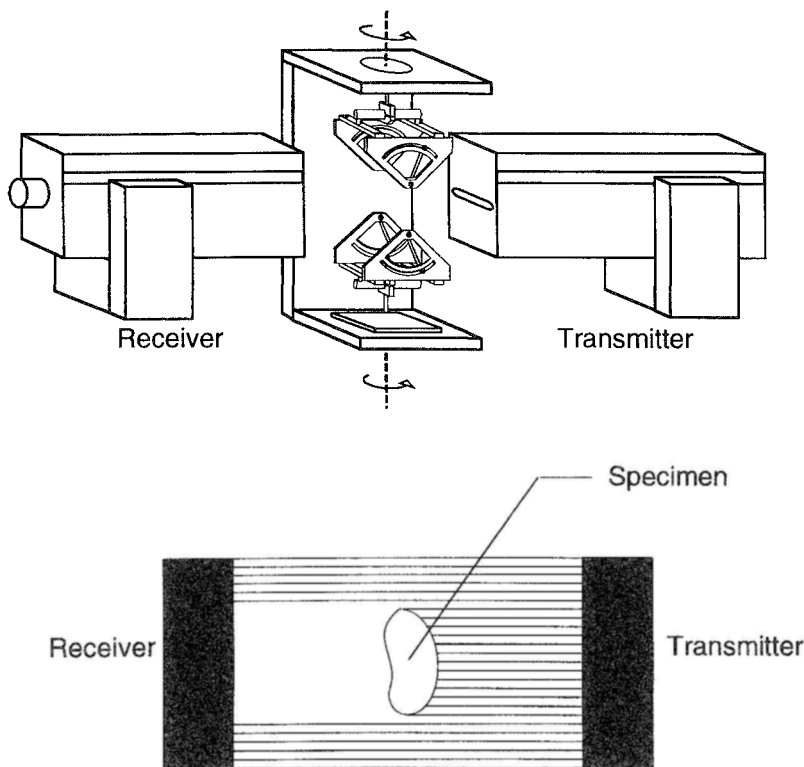


FIGURE 3. Schematic of the laser micrometer. (From Livesay, G. A., et al., *Anatomy and biomechanics of the human posterior cruciate ligament*, in *Clinical Biomechanics and Related Research*, Livesay, G. A., et al., Eds., Springer-Verlag, Tokyo, 1994, 200. With permission.)

stain. The specimen is then elongated in a materials testing machine while a video image of the specimen is captured. Using the VDA system, the videotape is played back and threshold “windows” placed over each reference line. The VDA hardware is able to track these thresholded lines and generate a continuous output voltage depending on the distance between the lines. Dividing the varying output voltages by the initial output voltage, the strain present in the tissue at any particular time can be calculated.¹⁹

A second system used is the Motion Analysis System (Motion Analysis, Santa Rosa, CA).^{21–23} Consisting of a CCD camera and an image processing system, a video image file is captured from each of the three cameras over time. Marks made on the tissue using high contrast stain can be tracked over time by thresholding their outlines and then tracking these outlines over time. Ease of use as well as a reduction in data analysis make this an attractive system for non-contact strain measurement.²³

Some additional advantages of a non-contact system are that midsubstance strains can be measured independently of those at the insertion sites. In addition, with careful stain marking, regional variation of strain can be measured.²³

C. CLAMPING OF TESTING SPECIMENS

The ligament or tendon to be tested has to be mounted in the tensile testing machine in such a way that the specimen is gripped without slippage at the clamps. In the past, many different methods have been devised for gripping directly to ligament and tendon tissue including sinusoidal²⁴

and cryo-clamp designs.²⁵ Even when this slipping is prevented, however, the direct clamping of ligamentous tissue causes stress concentrations which may introduce premature failure at the clamps. These problems can be avoided by leaving the ligament or tendon insertions to bone intact, thus allowing the specimen to be gripped on the bone substance and eliminating the aforementioned difficulties.¹⁹

On the other hand, when the bone-ligament-bone complex fails, there are three main modes of failure. The first is a frank ligament or tendon substance tear. The second is by bony avulsion, where the bone adjacent to the insertion site fails, the ligament remaining attached to bony debris. The third is by soft tissue pullout at the epiphyseal region with no bony involvement.

D. EFFECTS OF SPECIMEN ORIENTATION

The structural properties of this bone-ligament-bone complex are very dependent on the direction of the applied load during testing. For instance during testing of the canine and rabbit femur-anterior cruciate ligament-tibia complex (FATC), it was shown that the structural properties of the ACL changed with knee flexion angle being greatest at 0° of flexion and the least at 90° of flexion.^{26,27} We felt the differences are related to the uniformity of load distribution across the specimen. As our data for the rabbit FATC further revealed, load was either applied along the axis of the ACL (even load distribution) or along the axis of the tibia (uneven load distribution). For the specimens loaded along the ligament axis, structural properties were not dependent on knee flexion angle and most failures occurred by bony avulsion. In the specimens loaded along the tibial axis, structural properties varied with flexion angle and most failures occurred in the ligament midsubstance.²⁸

E. STRAIN RATE

In addition to specimen orientation, another consideration is the strain rate with which the specimen is tested. In a study of both rabbit ACLs and patellar tendons, the mechanical properties were shown to vary with strain rate, although these differences were relatively small compared to other factors.^{29,30} For example, a strain rate increase from 0.15%/sec. to 222%/sec. showed only minor effects on the resulting load-elongation and stress-strain curves. In the case of a higher strain rate, some increase in the tensile strength of the specimens was noted.²⁹

Many experimental factors contribute to the behavior shown by ligaments and tendons during testing, and careful attention must be paid to ensure elucidation of the correct mechanical properties.

IV. BIOLOGICAL FACTORS

It is well documented in the literature that the morphological, biomechanical and biochemical properties of soft tissue are sensitive to the tissue's environment. Factors such as maturation and age, immobilization and exercise, and especially the structure's anatomical location and functional role in the body will result in different properties in a ligament or tendon.

A. EFFECTS OF ANATOMICAL LOCATION AND FUNCTIONAL ROLE

Although their biochemical compositions are almost identical and their morphologies similar, the reported mechanical properties for ligaments and tendons in the literature vary considerably. For example, the ultimate strain values for ligaments, which can range from 12% to over 50%, tend to be somewhat larger than those for tendons, which have been reported to range from 9 to 30%.^{13,31-34}

A major factor contributing to this variability is the species and the anatomical location. For example, ultimate strain values on the order of 10–12% have been measured in the rabbit MCL

and ACL,³⁴ the swine digital flexor tendon³⁵ and the tendons of the equine foreleg.³⁶ These values vary, however, as ultimate strains have been reported of 8.1% in equine superficial digital flexor tendon,³⁷ 6% in the swine digital extensor,³⁵ and just 1.6% in the ligamenta flava of pig lumbar spines.³⁸

B. EFFECTS OF MATURATION AND AGE

The effects of aging and maturation on soft tissues, including skin, ligament and tendon, are well known. In general, it appears that biomechanical properties of ligaments and tendons improve rapidly as the animals reach skeletal maturity. Vogel and Morein observed that ultimate load, as well as Young's modulus and tensile strength, increase during early maturity of the rat tail tendon.^{39,40} Similarly, in the rabbit MCL, rapid increases were observed in cross sectional area, stiffness, and ultimate load.^{20,41} An important finding to note is the mechanism of failure also changed with skeletal maturity; because the epiphyses of these young animals are not closed, failure of the ligament most often occurs by tibial avulsion. On the other hand, once the epiphyses are closed, the ligament is most likely to fail at its midsubstance.⁴¹

After skeletal maturity is reached, however, little change in the structural properties of the rabbit femur-MCL-tibia complex (FMTC) occur, even after senescence.⁴¹ The structural properties, as well as the modulus, were almost constant after 12 months of age and decreased only slightly in the older animals. The human femur-ACL-tibia complex (FATC), on the other hand, does not follow this trend, as studies performed in our research center and others have demonstrated a significant decrease in structural properties of the FATC with age.^{26,42}

C. EFFECTS OF IMMOBILIZATION AND EXERCISE

The effects of joint immobilization, as well as exercise, cause profound changes in ligament and tendon properties. It has been demonstrated that stress deprivation can result in pannus formation and cartilage necrosis in high-contact regions,⁴³ while causing erosion of cartilage in non-contact areas.⁴⁴

The effects of immobilization followed by remobilization have been investigated in our research center.⁴⁵ Tensile testing of rabbit femur-MCL-tibia complexes was performed after nine and 12 weeks of immobilization as well as nine weeks of immobilization followed by nine weeks of remobilization. It was found that the nine and 12 weeks immobilized groups had ultimate loads of only 31% and 29%, respectively, of the contralateral controls ($p < 0.01$), with all specimens failing by tibial avulsion. In the remobilized group, the mechanical properties of the MCL substance returned nearly to control values. However, structural properties of the FMTC remained inferior to the controls and the mode of failure was still by tibial avulsion.

A study by Newton et al. has reported that the cross-sectional area of the rabbit ACL is also significantly decreased after nine weeks of immobilization. He found no significant differences in mechanical properties, though he did note a 32–40% increase in strain in the immobilized joints.⁴⁶

Interestingly, while stress deprivation causes profound detrimental effects in a relatively short amount of time, the positive contributions of exercise to the biomechanical properties of soft tissue are much less significant. Tipton and colleagues investigated this effect, observing a decrease in ligament water content, as well as a loss of waviness in collagen fibers, immediately post-exercise.⁴⁷ Subsequent studies have found minimal improvement of structural properties of bone-ligament-bone complexes after exercise.^{48–53}

In our research center, we have found that the effects of exercise on ligaments and tendons may be dependent upon the soft tissue structure as well as its anatomical location. The effects of life-long exercise and concomitant aging on the mechanical properties of the MCL were evaluated in the beagle.⁵⁴ Nine animals were exercised at 3 km/hr, 75 min./day, five days/wk, while wearing

an 11 kg backpack, for 9–12 yrs. Sedentary control groups were also used to evaluate the effects of aging. While aging was found to significantly reduce the tensile strength and strain at failure of the MCL substance, exercise was found to induce no significant differences in either the structural properties of the FMTC or the material properties of the ligament substance.

We also compared the effects of short term (3 months) and long term (12 months) exercise in the swine digital extensor and flexor tendons.^{35,55} Animals were exercised by running at the speed of 6–8 km/hour for a total of 40 km/wk. In the extensor tendon of the forepaw, exercise had no significant effects on mechanical properties in the short term, but long term increases in cross-sectional area and tensile strength over those of age-matched, non-exercised controls were observed. On the other hand, in the flexor tendon, there were no significant effects on its mechanical properties nor cross-sectional area of the tissue substance, but there was a 19% increase in ultimate load which could be attributed to an increase in strength of the tendon-bone junction.

For the femur-MCL-tibia complex from these animals, when the specimens were subjected to tensile tests to failure, there was a 38% increase in ultimate load and 14% increase in stiffness, though these changes were not statistically significant. The modulus, tensile strength and ultimate strain of the MCL substance also increased slightly though not significantly.⁵⁶ In addition, no changes in collagen or elastin concentration were observed between the two groups.

In general, there is a highly nonlinear relationship between levels of stress and ligament properties (Figure 4).⁵⁷ Immobilization can significantly compromise both the structural properties of the bone-ligament-bone complex as well as the mechanical properties of the ligament substance. Exercise, on the other hand, only moderately enhances ligament properties, even over the course of many years.⁵⁴

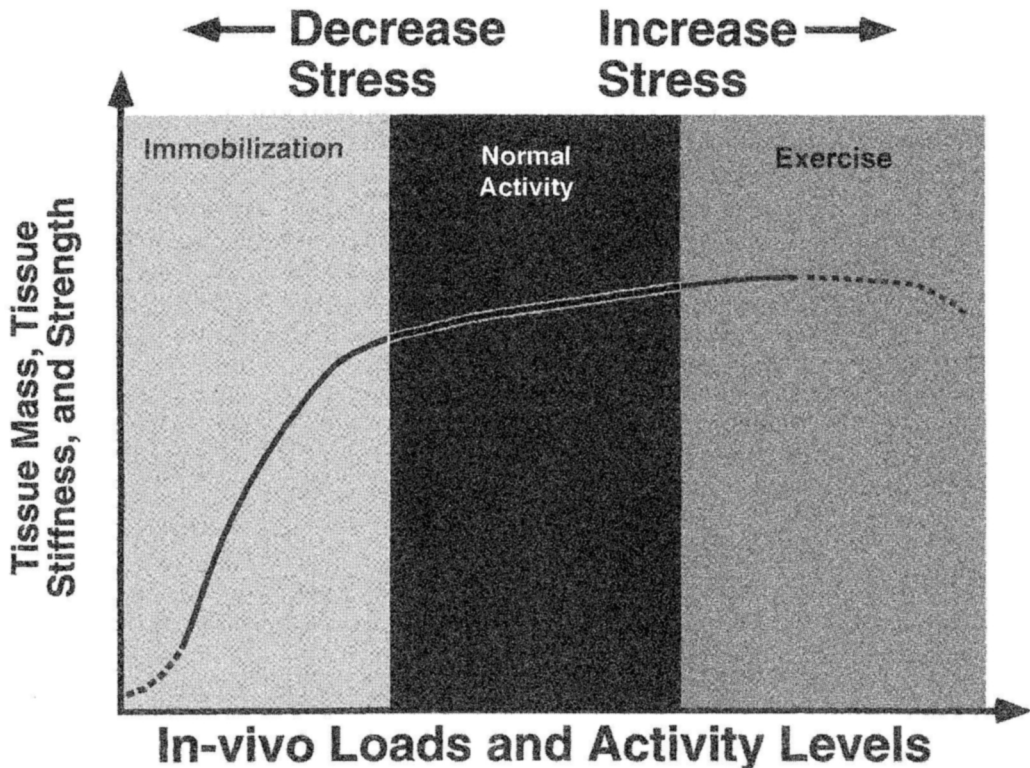


FIGURE 4. Hypothetical response of ligaments to levels of stress.⁵⁷

V. ENVIRONMENTAL FACTORS

A. EFFECTS OF DEHYDRATION

Because 65–70% of the composition of ligaments and tendons is water, it can be expected that their mechanical properties will vary with the moisture content in the structure. Some investigators have tested soft tissues in air, both with and without a saline drip applied, while others have immersed the specimen in a temperature controlled saline bath. Chimich et al. observed that for the rabbit MCL, there was an increased stress relaxation in response to cyclic loading when more moisture is contained in the ligament.⁵⁸ It has also been observed that the human patellar tendon will exhibit a greater elastic modulus and tensile strength when tested in a saline bath than with a drip solution applied.⁵⁹ For these reasons, it is very important that soft tissue be kept hydrated and that conditions be noted when testing ligaments and tendons.

B. EFFECTS OF TEMPERATURE

Another factor which may contribute to variability in results between studies is the temperature at which the soft tissues are tested. Numerous investigations have presented the data on biomechanics of ligaments and tendons when the tests were done at room temperature, while other data have been based on a temperature controlled bath environment.

Investigators have studied the relationship between temperature and the tensile properties of ligaments and tendons, with varying results. Rigby et al. reported no significant differences in mechanical properties of ligaments when testing between 0° and 37°C,⁶⁰ while others have reported a decline in elastic modulus and stiffness with increasing temperature.^{61,62} In our research center, the canine femur-MCL-tibia complex was tested in a saline bath at temperatures varying from 2–37°C under cyclic loading.⁶³ Each specimen was tested over the range of temperatures. This allowed the effects of the temperature change to be evaluated within each specimen, thus minimizing intra-specimen variability. It was found that an inverse relation exists between stiffness and temperature, and that the ligament relaxed to lower values under cyclic loading with higher temperatures. It should be noted that more than one hour of resting between tests was required for the ligament to return to its untested resting characteristics because of the viscoelastic nature of the tissue. These findings demonstrate the importance of both controlling and reporting temperatures when performing biomechanical testing.

C. EFFECTS OF FREEZING, STORING AND THAWING

Because it is often practically necessary to store specimens prior to testing or to use the specimens as allografts for reconstruction, the effects of freezing and storage on these tissues is of great interest. Several studies investigated the effect of post-mortem storage on tissue properties, but with conflicting results.^{64–67} It has been reported that the rabbit ACL becomes “less extensible” just one hour after death,⁶⁵ while others have found no changes up to 96 hours later in the rabbit FATC.⁶⁶ Authors have reported no significant effects of freezing on the structural properties of monkey ACLs⁴² while Dorlot et al. found an increase in the stiffness of the canine ACL.¹³

In our research center, the rabbit femur-MCL-tibia complex was used to study the effects of storage at –20°C for 1–3 months.⁶⁸ In order to protect the tissue from dehydration, the muscle and soft tissue were left intact, wrapped in saline-soaked gauze, and sealed in airtight plastic bags. Prior to testing, specimens were thawed overnight in the refrigerator (4°C). Previously frozen FMTCs were subjected to cyclic loading and then tensile loaded to failure. The same testing protocol was used on the contralateral fresh controls. No changes in cyclic stress relaxation, ligament cross-sectional area, ultimate load, ultimate deformation or energy absorbed to failure were observed after this method of freezing and storage. In addition, the mechanism of failure was the same in all specimens, suggesting that freezing had no significant effects on ligament properties, as well

as its insertion sites. However, during the initial loading and unloading of the FMT complex, a significant decrease in the area of hysteresis was observed in the frozen specimens as compared to the fresh specimens. Thus, we recommend that care be exercised in the storage of ligament and tendons in order to preserve their biomechanical properties.

VI. VISCOELASTIC PROPERTIES OF LIGAMENTS AND TENDONS

Because of their complex collagen and protein ultrastructure, ligaments and tendons display both time- and history-dependent viscoelastic properties. Uniaxial tensile testing of a ligament will exhibit a hysteresis loop as the unloading portion will not follow that of the loading portion, an indication that there is energy dissipation (Figure 5). Ligaments and tendons also exhibit the phenomena of creep and stress relaxation. When a constant load is applied to the ligament, the deformation increases over time, known as creep (Figure 6A); when a constant deformation is applied, a decrease occurs over time which is known as stress relaxation (Figure 6B).⁶⁹ This viscoelastic response has important physical and clinical implications.^{70,71} During cyclic loading and unloading, there is also a corresponding cyclic stress relaxation. Cyclic stress relaxation may help to prevent fatigue of ligaments when a large number of cyclic loads are applied, such as would occur when jogging. Conversely, stretching or prolonged exercising may enlist a gradual creep within a ligament or tendon. These effects manifest themselves clinically as temporary softening and increased laxity in joints after exercising. However, after a period of rest, these tissues can recover and return to their original lengths such that the joint returns to its normal stiffness.

VII. DETERMINING THE FUNCTIONAL ROLE OF LIGAMENTS/TENDONS

Characterizing the structural properties of the bone-ligament-bone complex and the mechanical properties of the ligament or tendon substance can aid in the understanding of the functional role of these tissues. By function, we focus on the contribution of ligaments and tendons to joint kinematics, as well as their forces *in situ*, when external loads are applied to the joint. Canine, rabbit, goat, and monkey models are among those which have been used to investigate the functional role of the ACL or MCL in the knee, as well as the effectiveness of various reconstructive techniques in restoring knee kinematics to as normal as possible.⁷²⁻⁸¹

Studies are sometimes performed in which an external load is applied and the resulting joint kinematics are recorded. However, it is important to recognize that constraining the joint motion in one or more degrees of freedom will yield vastly different data on joint kinematics.^{72,78,82}

In our research center, the anterior tibial translation in response to a 110 N anterior tibial load was determined in both 1 and 5 degrees of freedom (DOF) in the porcine knee.⁸² In 5 DOF, the anterior tibial loading of the unconstrained knee was 1.4 ± 0.2 times greater than of the constrained knee at 30° of flexion and 1.3 ± 0.1 times greater at 60° and 90° of flexion. This effect was significant for all flexion angles.

In order to demonstrate the complex roles of the ACL and MCL in restraining varus/valgus rotation, our research center had applied a varus-valgus bending moment to the canine knee at 90 degrees of flexion in three and five DOF.⁷² It was observed that with 3 DOF, varus-valgus knee laxity increased 171% after sectioning the MCL. However, when an identical test was performed in 5 DOF, it was found that VV laxity increased only 21%. This effect was attributed to the axial tibial rotation which is coupled with varus-valgus rotation and the results indicate that the ACL may compensate for the MCL under this loading condition when it is injured. The results of these studies indicate the importance of allowing multiple DOF motion in kinematic tests.

Also of interest when investigating the function of ligaments in a joint are the *in situ* forces within the ligament when an external load is applied. A number of methods have been used to

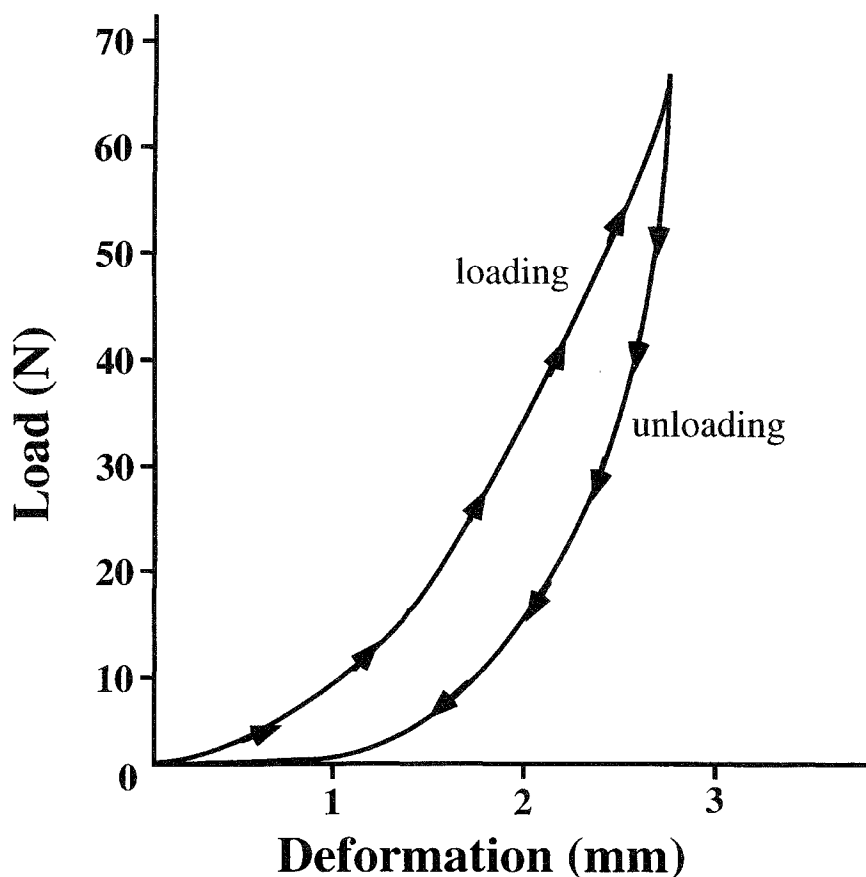


FIGURE 5. Typical hysteresis loop of a ligament subjected to tensile testing.

measure these forces in both animal and human models. Methods used have included the buckle transducer,^{83–85} and implantable transducers in the ligament midsubstance.⁸⁶ In our research center, kinematic linkages⁸⁷ have been used to measure length changes in the ACL which are then used to calculate the *in situ* force by correlating the changes with those of length-tension data of the FATC.

Recently, a Universal Force Moment Sensor (UFS) was used in combination with an Instron material testing machine in order to measure and compare the *in situ* forces in the ACL of goats, pigs, sheep and humans.⁸⁸ The magnitude and direction of the *in situ* force in the anteromedial and posterolateral bundles of the ACL was determined with the knee at 90° of flexion in response to an A-P load in 1 DOF. The results indicate that both the magnitude and direction of the *in situ* force in the ACL of the sheep were significantly different from that of the human; the porcine model was the only specimen not significantly different from the human, not only in the magnitude and direction of the total ACL force, but also that in its two bundles.

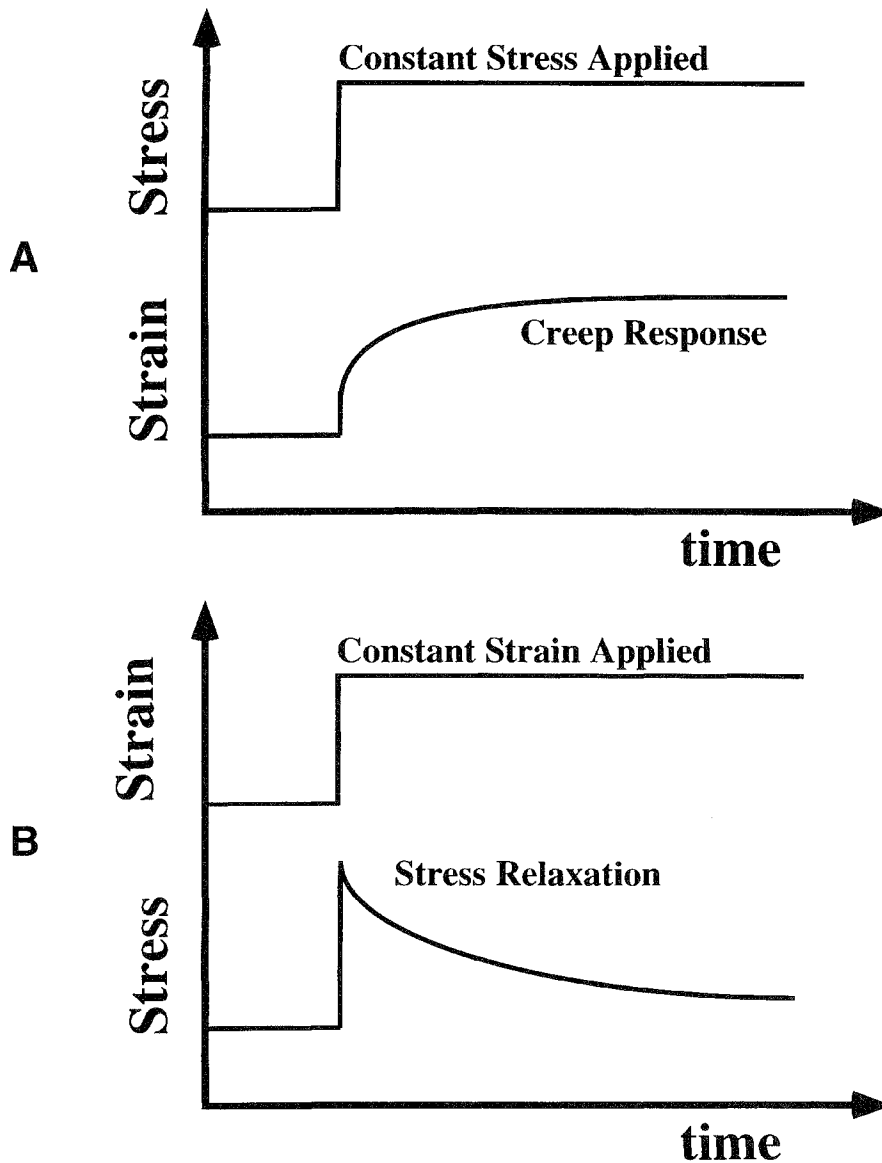


FIGURE 6. Schematic demonstrating (A) creep response (increasing deformation over time under a constant load) and (B) stress relaxation (decreasing stress over time under a constant deformation).

A. THE ROBOT/UFS TESTING SYSTEM

We have developed a new and unique testing system which combines a robotic manipulator with a universal force/moment sensor in order to measure the *in situ* forces in ligaments as well as determine joint kinematics (Figure 7). The robot (Unimate, Puma-762) is a six DOF, position-controlled device which can also perform in a force-controlled mode via force-feedback from

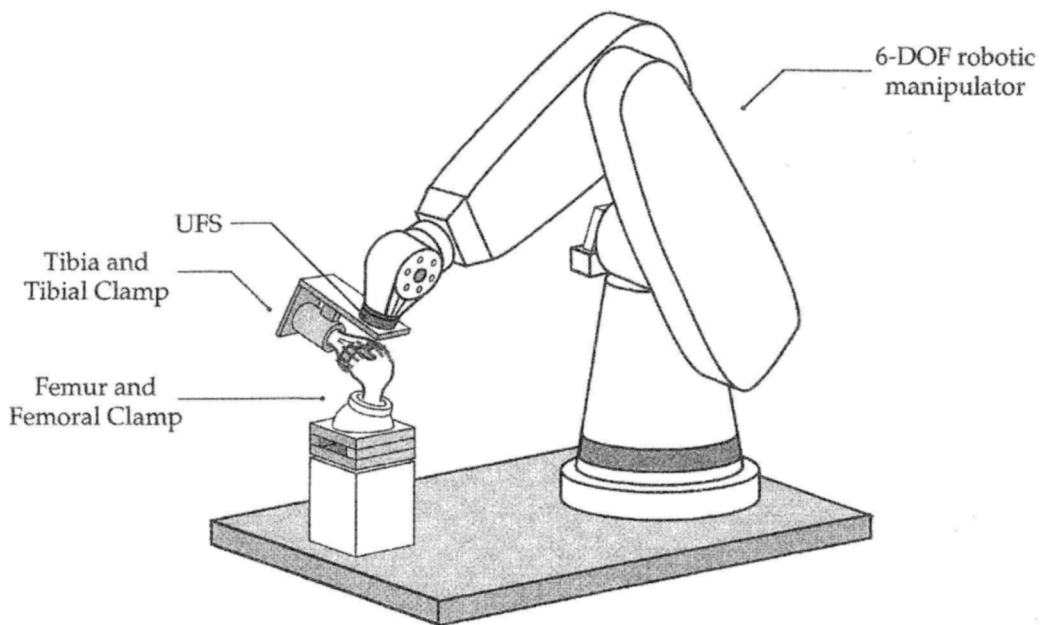


FIGURE 7. A schematic diagram of robot/UFS test system with a knee specimen in place.⁸²

the UFS. The UFS (JR3, model 4015) is capable of measuring three forces and three moments along its Cartesian axes. The UFS guides the robot to “learn” an exact path of motion in a specimen under force control and the robot later repeats the learned positions (path of motion) under position control.⁸⁹ The system offers a highly accurate, non-contact method of measuring the *in situ* forces in ligaments. When a ligament is sectioned, under position control the previously determined kinematics are repeated. At this point, the UFS is now used for data acquisition, as it measures the resulting forces after sectioning the ligament. The vector decrease in forces measured by the UFS yields the *in situ* forces of the ligament using the principle of superposition. The direction and point of application of the force can also be determined.

In addition to measuring *in situ* forces, the system can also be used to evaluate the changes in kinematics which occur after a structure is sectioned. By using force control to apply an external load to the joint before and after sectioning, the resulting kinematic changes in 5 DOF (flexion is fixed) can be determined. A further advantage is that all effects, such as that of sectioning a ligament or of a reconstruction, can be tested in the same knee. This minimizes interspecimen variability, making statistical comparisons more powerful and reducing the number of specimens needed.

A number of animal studies have been performed using the robot/UFS testing system.^{82,89–92} We have found the porcine knee to be an excellent model. The anatomy, size and geometry of the joint are comparable to those of the human knee, and the knees are easily and inexpensively obtained. Using this model, the effect of axial compression and anterior-posterior tibial loads on the *in situ* forces in the ACL and knee kinematics was evaluated. It was observed that the addition of a 200 N axial compressive load to a 100 N anterior-posterior load significantly increased anterior tibial translation of the knee, but decreased posterior tibial translation. It also caused a significant increase in the *in situ* forces in the ACL at 30°, 60° and 90° of flexion.⁹⁰

The porcine model has also been used to test the effect of tunnel placement for ACL and PCL replacement grafts on graft forces and knee kinematics.^{91,92} A 110 N anterior/posterior

load was first applied to the intact knee at 30°, 60° and 90° of flexion and the intact knee kinematics and ACL or PCL *in situ* forces determined. The ligament under investigation was then reconstructed with a BPTB graft with tunnels drilled in several locations. It was found that the ACL replacement graft was most successful in restoring normal AP kinematics when the tibial tunnel is drilled proximally as compared to a more central or distal fixation. In the PCL reconstructed knee, no significant difference in kinematics was observed with a proximal or anterior placement of the femoral tunnel; however, it was observed that the anteriorly-placed tunnel better replicated the trend of increasing *in situ* forces in the PCL occurring in the intact knee.

This system offers the potential of one day determining the *in vivo* forces in ligaments in animal models. If the *in vivo* kinematics of the joint are determined, the robot could later repeat these kinematics on the joint and thus determine the forces in the ligaments *in vivo*.

VIII. SUMMARY AND CONCLUSION

There have been many recent advances in the biomechanical testing of ligaments and tendons. The measurement of these properties is important to evaluate both reconstruction strategies and healing processes. However, there are many factors affecting the outcome of these measurements and readers are cautioned to evaluate the various experimental and biological factors in their interpretation of the published experimental data. Fortunately new bioengineering technologies are being developed such that it should be possible to standardize the mechanical testing of ligaments and tendons. By constantly building on past work, mechanical testing of soft tissue will enjoy further standardization as well as quality data management.

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Part III

Animal Models of Bone Conditions



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11 Animal Models of Bone Fracture or Osteotomy

Yuehuei H. An, Richard J. Friedman, and Robert A. Draughn

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I. INTRODUCTION

The processes of normal and abnormal fracture healing and the various factors affecting them have been widely studied since the days of Galen and Hippocrates. Today, with advanced technology and the desire of finding new solutions for fracture related problems, this field is still the object of numerous investigations. In the 1940s, Urist and Johnson¹ mentioned that there were more than 4,000 publications on this subject already in the literature, and this figure has been growing since then. One of the most important elements in studies of fracture healing or fracture fixation is establishing a standard method to make a reproducible fracture, which would allow results from

different centers to be compared. Numerous models of experimental fractures have been published. This chapter attempts to summarize them and to pave an easier road for researchers to find valid models for new projects.

II. FRACTURE HEALING PROCESS

Normal fracture healing undergoes three phases: inflammation, reparation, and remodeling.²⁻⁴ At the inflammatory stage, hemorrhage and cell death causes an inflammatory response. It evolves into granulation tissue with mesenchymal fibroblasts, macrophages, and lymphocytes. The granulation tissue absorbs dead tissue and provides chondrogenic and osteogenic precursor cells, forming the foundation for further healing phases. During the reparation phase (external callus formation), the mesenchymal cells in the granulation tissue undergo rapid chondrogenesis, followed by endochondral ossification supplemented by appositional bone formation. The fracture callus formed during this phase provides stability for osteogenesis. The remodeling phase includes gradual resorption of the periosteal bony callus, maturation of bone structure and the restoration of the cortical bone structure.

There are three main types or modes of fracture healing mechanisms depending on the rigidity of fracture fixation:⁴ (1) direct early healing (primary osteonal healing); (2) direct healing with substantial callus formation (secondary osteonal healing); and (3) non-osteonal healing by periosteal and endosteal callus formation. The three phases of fracture healing are closely correlated to the primary and second osteonal healing mode. The rigidity of immobilization or fixation of the fracture site determines the amount of cartilaginous callus formation and, consequently, determines the healing mode. The greatest amount of callus is seen in nonfixed unstable fractures, the smallest amount of chondral tissue in rigid fixed fractures (such as compression plating), and an intermediate amount of callus formation occurs in most less rigid fixed fractures (such as intramedullary canal nailing, external fixation, and less rigid plating).

III. ANIMAL MODELS OF DIAPHYSEAL FRACTURES

A. MODELS OF DIAPHYSEAL FRACTURES

Major diaphyseal fracture models are listed in Table 1. Several more diaphyseal models are included in Chapter 12 and they were used for testing bioabsorbable fixation devices. Different animals have been used, ranging from small to large or from low to high vertebrate animals including the mouse, rat, rabbit, cat, dog, sheep, and goat. The most commonly used are rat, rabbit, dog, and sheep.

There have been four major fracture-producing methods for diaphyseal fracture models, including (1) manual fractures;^{7,17-19,77} (2) three-point bending methods, such as those fracture devices described by Ekeland,^{8,75} Bak and Andreassen,^{14,79,80} Tepic et al.⁷² and Greiff;²¹ (3) a guillotine-like fracture apparatus, including those described by Jackson,⁸ Sarmiento,¹⁰ Northmore-Ball,⁷⁸ Bonnarens and Einhorn,¹² and An et al.;²⁸ and (4) osteotomies using a saw or scissors.^{29,79} The first three methods are more natural, mimicking accidental fractures.

Most diaphyseal fractures can be fixed with internal fixation such as intramedullary rods or pins, plate and screws, screws only, thread or wires, alone or in combination. Intramedullary pins can be introduced percutaneously in rat models to create a closed fracture. Plate and screws are often used in larger animals. One or two screws can be used to fix an oblique diaphyseal fracture, but external supports are often needed for a satisfactory fixation. Fixation may not be used in small animals such as mice or rats to facilitate a natural healing process or in the cases of radial, ulnar, or fibular fracture models because of the existing support from the companion bones, ulna, radius, or tibia. External fixators are often used for larger animals such as dogs or sheep. Casts or splints are sometimes used alone or for extra protection after internal fixation.

TABLE 1
Diaphyseal Fracture Models in Animals

Animal	Bone	First author ^{ref.}	Fracturing method	Fracture type	Fixation method	Purpose or treatment
Mouse	Tibia	Hsu 1969 ⁵	Manual	T*	No fixation	Study of fracture healing
		Hiltunen 1993 ⁶	Impact device	T/O†	Intramedullary pin	Study of fracture healing
Rat	Femur	Eskelund 1950 ⁷	Manual	T	No fixation	Study of fracture healing
		Jackson 1970 ⁸	Jackson's punch	T	Intramedullary pin	Study of fracture healing
		Kernex 1973 ⁹	Manual	T	No fixation	Study of fracture healing
		Sarmiento 1977 ¹⁰	Jackson's punch	T	Intramedullary pin	Effect of weightbearing on fracture healing
		Ekeland 1981 ¹¹	Fracturing forceps	T	No fixation	Study of fracture healing
		Bonnaren 1984 ¹²	Impact device	T	Intramedullary pin	Study of fracture healing
		Huo 1991 ¹³	Manual	T	Intramedullary pin	Effect of ibuprofen on fracture healing
		Grundnes 1993 ¹⁴	Saw, osteotomy	T	Intramedullary pin	Study of fracture healing
		Olmedo 1994 ¹⁵	Three point bending clamp	T	Metal catheter	Drug delivery to fracture site
		Hietaniemi 1996 ¹⁶	Saw, osteotomy	T	Unstable fixation	Creation of non-union
	Tibia	Urist 1941 ¹⁷	Manual	T	No fixation	Study of fracture healing
		Penttinen 1972 ¹⁸	Manual	T	No fixation	Study of fracture healing
		Hulth 1964 ¹⁹	Manual	T	No fixation	Effect of cortisone on fracture healing
		Greiff 1978 ²⁰	Fracturing forceps	T	Intramedullary pin	Study of fracture healing
		Greiff 1978 ²¹	Fracturing forceps	T	Intramedullary pin	Study of fracture healing
		Molster 1982 ²²	Saw, osteotomy	O	Intramedullary pin	Effect of instability on fracture healing
		Lowe 1983 ²³	Guillotine device	T	Intramedullary pin	Study of fracture healing
		Bak 1988 ²⁴	Fracturing forceps	T	Intramedullary pin	Study of fracture healing
		Aro 1989 ²⁵	Manual	T	Intramedullary pin	Study of fracture healing
		Keller 1993 ²⁶	Saw, osteotomy	T	Plate and screws	Effect of prostaglandin on fracture healing
		Nyman 1993 ²⁷	Manual	T	Intramedullary pin	Effect of clodronate on fracture healing
		An 1994 ²⁸	Guillotine device	T	Intramedullary pin	New fracture device
		Nielsen 1994 ²⁹	Fracturing forceps	T	Intramedullary pin	Effect of TGF- β on fracture healing
		Otto 1995 ³⁰	Special pliers	T	No fixation	New fracturing method
		David 1996 ³¹	Ring-cutter saw	T	Intramedullary pin	Effect of laser on fracture healing
Fibulae		Herbsman 1966 ³²	Scissors	T	No fixation	Study of fracture healing
		Kawaguchi 1994 ³³	Bone cutter	T	No fixation	Effect of bFGF on fracture healing
Radius		Volpin 1986 ³⁴	Scissors	T	No fixation	Study of fracture healing

TABLE 1
Diaphyseal Fracture Models in Animals

Animal	Bone	First author ^{ref.}	Fracturing method	Fracture type	Fixation method	Purpose or treatment
Rabbit	Radius	Chai 1985 ⁵⁵	Osteotomy	T	No fixation	Effect of a herb on fracture healing
		Bushberg 1985 ³⁶	Bending with a metal bar	T	Splinting and tapping	Uptake of ⁶⁷ Ga and ^{99m} Tc MDP
	Femur	Manninen 1993 ³⁷	Osteotomy	T	SR-PLA rods	Use of bioabsorbable material on fracture fixation
	Tibia	Albanese 1996 ³⁸	Osteotomy	T	Plate and screws	Effect of osteotomy on bone growth
		White 1977 ³⁹	Osteotomy	T	External fixator	Effect of compression force on fracture healing
		Paavolaianen 1979 ⁴⁰	Osteotomy	T	Plate and screws	Effect of compression plate on fracture healing
		Ashhurst 1982 ⁴¹	C-clamp fracture device	T	Plate and screws	New fracture device
		Court-Brown 1985 ⁴²	Osteotomy	T	Ext. fixator/cast	Effect of fixation on blood flow
		Terjesen 1986 ⁴³	Osteotomy	T	External fixation	Effect of fixation stiffness on fracture healing
		Aalto 1987 ⁴⁴	Osteotomy	T	External fixator	Effect of rigidity of fixation on fracture healing
		Lafman 1989 ⁴⁵	Osteotomy	T	Rigid internal plate fixation	Studying stress shielding of the rigid fixation
Dog	Femur	Burr 1990 ⁴⁶	Impulsive loading	Micro	No fixation	New stress fracture model
		Carpenter 1992 ⁴⁷	Dental burr osteotomy	T	External fixator	Growth hormone on fracture healing
		Kaatinen 1993 ⁴⁸	Osteotomy	T	Intramedullary nail	Observation of healing patterns
		Jacob 1993 ⁴⁹	Drill and bone biter	T	Plate and screws	Local use of cefazolin to prevent infection
		Worlock 1994 ⁵⁰	C-clamp fracture device	T	DC plate or intramedul. nail	Effect of fracture stability on infection rate
		Nash 1994 ⁵¹	Osteotomy	V-shaped	Intramedullary PGA rod	Effect of PDGF on fracture healing
		Pienkowski 1994 ⁵²	Osteotomy	T	No fixation	Effect of electromagnetic stimulation on fracture healing
		Brighton 1991 ⁵³	Manual fracture	T	No fixation	Study of fracture healing
		Rhinelander 1983 ⁵⁴	Saw osteotomy	5-cm O	Pins and nylon straps	Effect of plain nylon straps on fracture fixation
		Miettinen 1992 ⁵⁵	Osteotomy	T	SR-PLA or SR-PGA	Fracture fixation with absorbable rods
		An 1997 ⁵⁶	Trephined	Circular	PGA/PLA screws	Fixation of cortical bone piece with absorbable screws

Tibia	Skirving 1987 ⁵⁷ Gilbert 1989 ⁵⁸	“Bone breaker” Osteotomy	T T	Plate and screws Different ext. fixators	Effect of different plates on fracture healing Effect of fixation stiffness on fracture healing
	Smith 1990 ⁵⁹ Tiedeman 1990 ⁶⁰ Markel 1990 ⁶¹	Osteotomy Osteotomy Osteotomy	T T T	Plate and screws External fixator External fixator	Effect of fixation on blood flow X ray assessing fracture healing Fracture healing assessed by QCT, SPA, DEXA, and MRI
Radius	Aro 1993 ⁶²	Osteotomy	T/60°O	External fixator	Effect of fracture stability, fracture type, loading on fracture healing
	O’ Sullivan 1994 ⁶³ Lenchan 1985 ⁶⁴ Chakkalakal 1990 ⁶⁵	Saw osteotomy Wire wheel puller Saw osteotomy	T T T	External fixator External splinting Pins between radius and ulna	Effect of weight bearing on fracture healing Effect of EHD [†] on fracture healing Study of fracture healing
Sheep	Peter 1996 ⁶⁶ Heitemeyer 1990 ⁶⁷	Three point bending by wire Triple wedge osteotomy	T C¶	Coaptation splinting Plate + screws, bridging plate intramedul. nail, or ext. fixator	Effect of alendronate on fracture healing Fixation methods for comminuted fracture
	Goodship 1993 ⁶⁸ Schemitsch 1994 ⁶⁹	Osteotomy Tepic’s fracture device	T Spiral	External fixator Intramedullary nail	Fixator frame stiffness on fracture healing Reamed and unreamed nailing on cortical blood flow
Goat	Wallace 1995 ⁷⁰	Osteotomy	T	External fixator	Serum angiogenic factor level after tibial fracture
	Schemitsch 1996 ⁷¹	Tepic’s fracture device	Spiral	Intramedullary nail	Reamed or unreamed nailing on soft tissue blood flow
Cat	Tepic 1997 ⁷²	Tepic’s fracture device	T	Point contact plate and screws	Testing effect of point contact fixator on bone healing
	Augat 1997 ⁷³ Curtis 1994 ⁷¹	Osteotomy Osteotomy	T T	External External or internal	Assessment of fracture by peripheral CT Effect of fixation method on infection
Calf	Hara 1994 ⁷⁵ Illi 1992 ⁷⁶	Saw osteotomy Saw osteotomy	T 45°	PLA intramedullary rods PLA or AO metallic screws	Fixation of femoral diaphyseal fracture Fixation of metacarpal diaphyseal fracture

* T = Transverse
† O = Oblique
‡ EHD[†] = Ethane-1,1-diphosphonate
C¶ = comminuted fracture.

B. AUTHORS' PREFERRED DIAPHYSEAL MODELS

1. Rat Tibial Fracture (A Tibia Model Developed in Authors' Laboratory)

When attempting to produce a standard closed fracture in the rat femur, we found it very difficult because the bulky soft tissues around the femoral bone made positioning of the rat thigh on the support anvil of the fracture apparatus very difficult. The rat tibia, on the other hand, is subcutaneously located and by its anatomic location better suited than the femur for the production of a closed fracture. So, modified from the method of Bonnarens and Einhorn,¹² a fracture apparatus has been built in the authors' laboratory (Figure 1). The support anvil was made with an adjustable foot rest, which ensures that all of the fractures are at the same level by positioning the rat leg on the anvil with the foot against the foot rest.²⁸

To test the apparatus, 88 SD rats (300–350 gm) underwent percutaneous bilateral intramedullary pinning prior to the production of a fracture using this apparatus. Under general anesthesia, a hole was made 4 mm proximal and 2 mm medial to the tibial tuberosity percutaneously using a 20-gauge needle (Figure 2). The needle was driven directly into the medullary canal, and by rotating it, reamed the canal to within 2–5 mm of the ankle joint. A 0.9 mm Kirschner wire was then placed down the intramedullary canal, and the end of the wire was cut as short as possible so that the skin could roll over. No stitches were needed. Reaming was a necessary part of the procedure to allow the wire to seat and prevent it from perforating the cortex.

Thereafter, the rat was placed supine and the lower leg was positioned on the anvil of the fracture apparatus in an abducted and externally rotated position and the guillotine blade was lined up with the middle portion of the lower leg (about 3–5 mm proximal to the junction of the tibia and fibula). The travel distance of the guillotine ramming system was set at 2.5–3.0 mm. The 500-gm weight was dropped from a height of 40 centimeters, driving the guillotine blade to fracture the tibia. The legs were then radiographed to examine the fracture and fixation.

Animals were sacrificed three or five weeks after the surgery and the fracture healing was evaluated by X ray (Figure 2), histological and mechanical methods. Using this method, the fracture site is easy to control because of the adjustable foot rest built into the support anvil. Mechanical testing and histological studies showed that a standard fracture healing process was obtained. It is concluded that this modified method creates a standard, reproducible transverse closed fracture of rat tibia.

2. Other Rat Fracture Models

Manual techniques for fracture production are no longer acceptable, as they have no controls over the force produced and there can be wide variations in the fracture site and configuration. The lack of fixation leads to variable degrees of displacement and motion of the fragments during the healing period, resulting in a slow healing process. Three-point bending using specially designed forceps^{11,24,78–80} and Greiff's fracture device²¹ are easy to use and control the location of the fracture site. However, there is not enough control built in, and the force applied remains unknown. The method described by Jackson⁸ can create a standard, closed, transverse, internally stabilized mid-shaft femur fracture in the rat with a minimal soft tissue injury. The fracture mechanism mimics the traumatic situation, but the fracture apparatus is complicated. The method described by Bonnarens and Einhorn¹² has the same advantages and the fracture device can be easily built. But the shortcoming of using the femur is that the fracturing procedure is difficult and sometimes frustrating, since the fracture site is not easy to control because of the bulky soft tissue around it.

To achieve rapid healing and avoid angulation and movement, open^{8,12,24,77} or closed^{77,79} intramedullary pinning, both before⁹ or after fracturing,⁷⁶ have been used successfully. The closed pinning technique used in authors' laboratory has been proved to be simple and reliable.²⁸

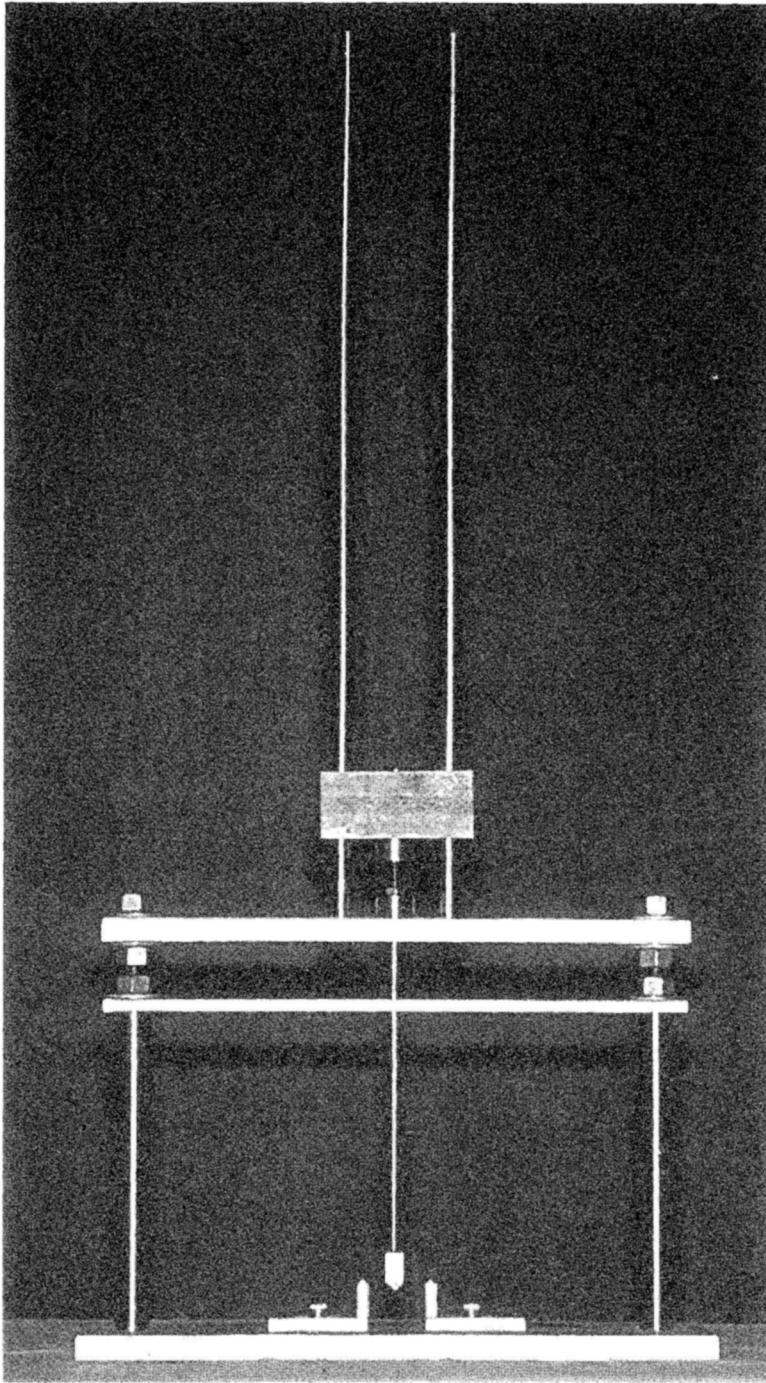


FIGURE 1. Photograph of the fracture device used in the authors' laboratory.

3. Tibial Fractures in Rabbits, Dogs and Sheep

Rabbit tibial fracture is another major fracture model (Table 1). The fracturing methods used in this model include saw osteotomy, fracture devices, and drill plus bone cutter. A high-speed dental burr also has been reported for creating a tibial osteotomy.⁴⁷ Compared to rat long bones,

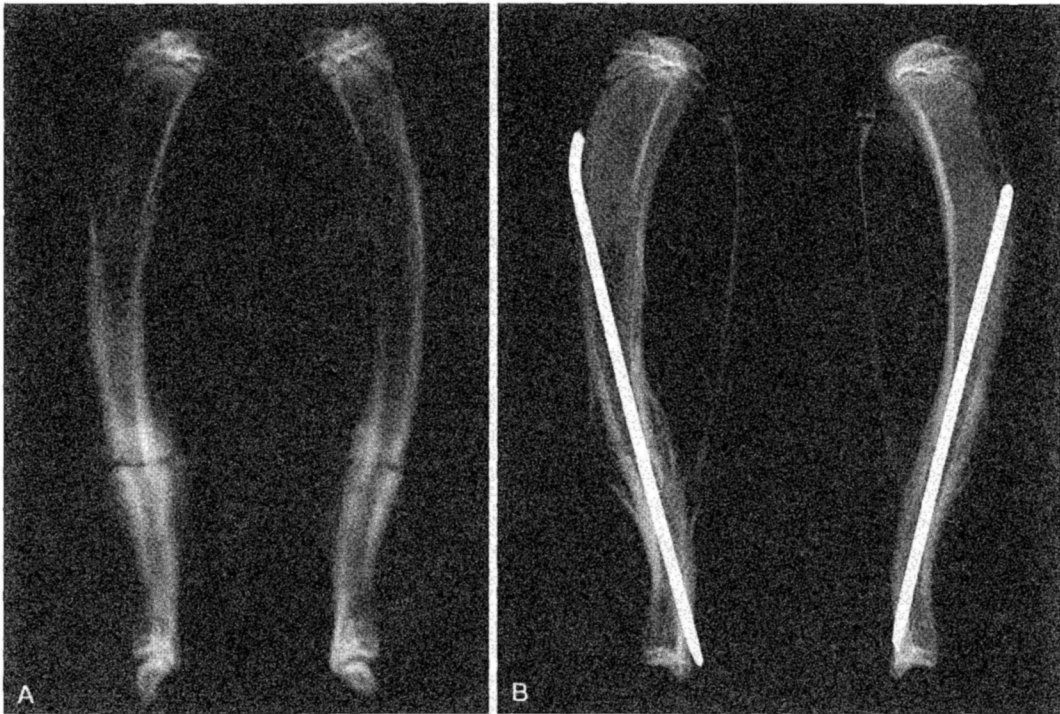


FIGURE 2. Lateral radiographic image of a rat tibia three weeks following intramedullary nailing and fracturing (A). Note the intramedullary nails were removed. The same type of fracture fully healed at five weeks (B).

rabbit tibia is suitable for different kinds of fixation methods, such as plate and screws, intramedullary nailing, external fixator, and even long-leg cast. This model has been used successfully for many purposes such as the evaluation of normal fracture healing, the effect of fixation devices, or effect of growth factors, hormones, or prostaglandin E_2 on fracture healing. A unique stress fracture model of rabbit tibia was created by using repeated application of nontraumatic impulsive loads for three to nine weeks.⁴⁶ Changes in the bone were monitored by radiograph and bone scan and the presence of stress fractures was confirmed in some cases at six weeks.

Aside from being higher level vertebrates, the unique side of dogs and sheep is that they have large size bones which allow the direct use of the fixation devices designed for human. Osteotomies have been created by using a saw, a “bone breaker,”⁵⁷ or Tepic’s fracturing device.⁷² The osteotomies have been fixed with plate and screws, intramedullary nails, or external fixators. These tibial fracture models have been used successfully for studying fracture healing, factors affecting bone healing, efficacy of fixation devices, and effects of fracture on bone blood flow (Table 1).

4. Radial Fractures in Rabbits and Dogs

Radial osteotomy in rabbits and dogs can be made using a powered saw. In the authors’ laboratory, a small diamond circular saw blade driven by nitrogen gas through a dental handpiece has been proven to be accurate and caused limited trauma to the bone and surrounding tissues in a rabbit model (Figure 3). A three point bending fracture device could also be used to create a fracture of the radius.⁶⁴ The advantage of this model is that no fixation is needed, especially in the rabbit, so that bone healing can be observed without the influence of invasive fixation devices. However, because of the active nature of dogs, ulnar fracture may occur. The risk of ulnar fracture can be minimized by the use of an external support such as a coaptation splint for extra protection.^{64,66} The fractured radius in dogs can also be transfixed internally to the ulna with Steinmann pins.⁶⁵

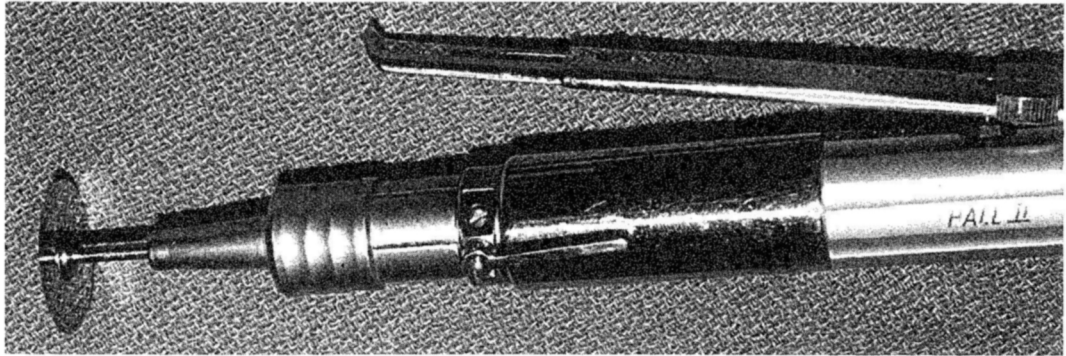


FIGURE 3. A small diamond circular saw used for creating radial osteotomies in the rabbit.

IV. ANIMAL MODELS OF EPIPHYSOMETAPHYSEAL MODELS

Many epiphysometaphyseal osteotomy models have been reported in the literature. Most of the models are used for testing bioabsorbable fixation devices (see Chapter 12). Only the models not used for testing absorbable materials and several representative ones used for testing absorbable materials are listed in Table 2, such as the femoral transcondylar osteotomy in the rabbit,⁸³ the osteotomy of medial tibial plateau in the rabbit,⁸⁷ Salter-Harris type IV fracture in the goat,⁹⁰ the subcapital femoral neck fracture in the sheep,⁸⁹ and the lateral femoral condyle osteotomy in the dog (see Figure 3 in Chapter 12).²⁸

V. ANIMAL MODELS OF DELAYED UNION AND NONUNION

Delayed union and nonunion are special types of fractures models, representing abnormal fracture healing. They are used to study physiopathological conditions and treatment methods of delayed union and nonunion. Delayed unions and nonunions of animal radius, femur, tibia and fibula have been reported in rats, rabbits, and dogs (Table 3).

The basic principle of creating a delayed union and nonunion is the depletion of osteoconductive and osteoinductive factors at the fracture site. The common methods include a 3–10 mm resection of the diaphyseal or metaphyseal bone and the extensive stripping of surrounding periosteum. External fixators have been used for fixing osteotomies and keeping the bone ends apart. The latter can be also achieved by using a silicone rubber spacer.⁹⁵ To devitalize the bone ends, freezing at -20°C has been reported.⁹⁸ Loose intramedullary nailing (by excessive reaming) also facilitate the establishment of delayed union or nonunion.^{16,91}

VI. EVALUATION METHODS

A. RADIOGRAPHY

Radiography is the basic method for evaluating fracture healing. Radiographs should be taken immediately after surgery to examine the location of the fracture and the quality of fixation. Periodic radiographies are essential for monitoring the process of fracture healing. After the animals are sacrificed, the bone specimens should be radiographed again using a high resolution X ray machine, such as Faxitron (Hewlett Packard, McMinnville, OR). The high resolution images may be used for a variety of measurements, such as bone density or bone dimensions. For comparison, it is very important that radiographs are taken before surgical procedures and also include the control limb.

For long bone fractures or osteotomies, healing parameters, such as periosteal reaction (callus formation), quality of union, and bone remodeling are quantitated based on a radiographic scoring

TABLE 2
Animal Models of Epiphysometaphyseal Osteotomies and Other Osteotomies

Animal	Bone	Osteotomy or fracture	Fixation treatment	Purpose	First author, year ^{ref.}
Rabbit	Femur	Transcondylar	PGA thread and rods	Testing absorbable fixation devices	Vainionpää 1986 ⁸³
		Salter-Harris III and IV fracture	Fixed with screw or no fixation	Testing effect of rigid fixation	Gomes 1993 ⁸⁴
	Tibia	Medial condyle	No fixation	Observation of bone remodeling	Bogoch 1993 ⁸⁵
Dog	Femur	Upper metaphyseal	No fixation	Simulating metaphyseal fracture	Aronson 1990 ⁸⁶
		Proximal tibial	PLA or stainless steel screws	Testing absorbable fixation devices	Matsusue 1991 ⁸⁷
	Pig	Lateral femoral condyle	PGA/PLA screws	Testing absorbable fixation devices	An 1997 ²⁸
Sheep	Femur	Lateral femoral condyle	No fixation	Fracture produced by shear force	Tomatsu 1992 ⁸⁸
		Subcapital osteotomy	SR-PLA lag-screws	Testing absorbable fixation devices	Vasenius 1993 ⁸⁹
Goat	Femur	Salter-Harris IV fracture	PLA, polydioxanone pins	Testing absorbable fixation devices	Domigian 1993 ⁹⁰

TABLE 3
Animal Models of Bone Nonunion

Animal	Bone	Method of fixation	Delayed union or Nonunion	Purpose or treatment	First author, year ^{ref.}
Rat	Femur	Reaming, manual fracture, nailing	Nonunion	New method to produce nonunion	Hietaniemi 1995 ⁹¹
		As above	Nonunion	Connective tissues in bone nonunion	Hietaniemi 1996 ¹⁶
Rabbit	Fibula	8 mm periosteum stripped at the distal fibula	Nonunion	New method to produce nonunion	Aro 1985 ⁹²
	Radius	10 mm defect, periosteum resection	Delayed union	Treatment with coupled electric fields	Rijal 1994 ⁹³
	Tibia	Osteotomy, marrow removing, silastic sheath	Nonunion	A new nonunion model	Oni 1995 ⁹⁴
		wrapping of bone ends			
Dog	Fibula	Silicone rubber spacer for 48 days	Delayed union	Treatment with direct electric current stimulation	Peterson 1983 ⁹⁵
	Radius	3 mm defect, 10 mm periosteum stripping, no fixation	Nonunion	New method to produce nonunion	Santos Neto 1984 ⁹⁶
	Radius	5 mm defect, 2 cm periosteum stripping, no fixation	Nonunion	New method to produce nonunion	Volpon 1994 ³⁴
	Tibia	6 mm defect, external fixator	Nonunion	Treatment with injectable demineralized bone matrix	Tiedman 1991 ⁹⁷
		5 mm osteotomy, external fixator bone ends frozen twice at -20°C	Nonunion	Prediction of nonunion with dual energy X ray absorptiometry	Markel 1995 ⁹⁸

TABLE 4
Radiographic Scoring System for Fracture Healing

Categories	Scores
Periosteal reaction	
Full (across the defect)	3
Moderate	2
Mild	1
None	0
Bone union	
Union	3
Moderate bridge (>50%)	2
Mild bridge (<50%)	1
Nonunion	0
Remodeling	
Full remodeling cortex	2
Intramedullary canal	1
No remodeling	0
Maximum total score	8

system modified from the ones by Bos et al.,⁹⁹ Lane and Sandhu,¹⁰⁰ Yang et al.,¹⁰¹ and Johnson et al.¹⁰²(Table 4).

B. HISTOLOGY AND HISTOMORPHOMETRY

Histology is another basic method for evaluating fracture healing. Generally, longitudinal sections through the fracture callous and surrounding area are cut and stained with H&E or other stains. Common histological parameters include the following categories: callus formation, bone union, marrow changes, and cortex remodeling, which can be semi-quantitated based on a scoring system modified from the ones by Bos et al.,⁹⁹ Nilsson et al.,¹⁰³ Lane and Sandhu,¹⁰⁰ Heiple et al.,¹⁰⁴ and Suh et al.¹⁰⁵ (Table 5). Parameters for histomorphometry include original and new bone area, chondral tissue, fibrocartilage, fibrous vascular tissue (marrow).¹⁰⁶ See Chapters 6 and 7 for more information on bone histomorphometry.

C. MECHANICAL TESTING

To evaluate the mechanical properties of bone associated with fracture healing, bending and torsional tests are the most popular. For bending tests, the classic article is the one reported by Burstein et al.¹⁰⁷ The bending test has been used to measure the mechanical properties of mouse tibia,⁶ rat femur,^{10,11} rat tibia,^{22,24,28,29} rabbit tibia,^{26,44,45–51} canine radius,^{64–66} canine tibia,^{57,58,61} and sheep tibia.^{67,72,73}

Torsional tests often are used for larger tubular bones such as the tibia or femur of rabbits^{39,40} and dogs.^{28,62,63} Based on torque-angle curves and radiographic findings, for mechanical stages of fracture healing were classified by White et al.:³⁹ I — fracture through the original fracture site (low stiffness); II — through the original fracture site (high stiffness); III — partially through the original fracture site and partially through intact bone (high stiffness); and IV — entirely through intact bone (high stiffness). These stages correlate with the quality of fracture healing and the healing time.

Indentation tests have been used to evaluate the mechanical properties of the fracture callus.⁶¹ Occasionally, tensile tests have been used for testing bone mechanical properties as a function of fracture healing.^{11,27}

TABLE 5
Histological Scoring System for Fracture Healing

Categories	Scores
Callus formation	
Full (across the defect)	3
Moderate	2
Mild	1
None	0
Bone union	
Full bone bridge (union)	3
Moderate bridge (>50%)	2
Mild bridge (<50%)	1
No new bone in the fracture line (nonunion)	0
Marrow changes	
Adult type fatty marrow	4
2/3 replaced by new tissue	3
1/3 replaced by new tissue	2
Fibrous tissue	1
Red	0
Cortex remodeling	
Full remodeling cortex	2
Intramedullary canal	1
No remodeling	0
Maximum total score	12

* For some osteogenic materials such as collagen sponge/GFs, this category may not apply because of the nearly absent periosteal reaction.

D. OTHER EVALUATION METHODS

Other methods or special devices have been used for evaluating fracture healing, such as autoradiography,²⁰ quantitative roentgenographic densitometry (QRD),^{60,61} single-photon absorptiometry (SPA),^{25,61} dual energy X ray absorptiometry (DEXA),^{61,98,108} bone scan (for osteotomies³⁶ or stress fractures⁴⁶), quantitative CT (QCT),^{61,73} or MRI.^{61,109} Biological markers for bone healing and bone formation can be detected in serum (see Chapter 6).

VII. METHODS FOR ENHANCING FRACTURE HEALING

This topic is too big for the size of this chapter. Readers may want to refer to the comprehensive review on this subject by Einhorn,¹¹⁰ who categorized the methods of enhancing fracture healing as biological enhancement and biophysical enhancement. The former includes local (osteogenic, osteoconductive, and osteoinductive methods) and systemic approach (prostaglandins and other circulating osteogenic substances). We want to add to the category of systemic approach the enhancing effect of certain hormones or drugs on fracture healing. The biophysical enhancements include local mechanical, electrical, and ultrasonic stimulation.

A. BONE GRAFTING

Bone grafting is an osteogenic approach including the use of autogenic or allogenic bone grafts, demineralized bone matrix (DBM) graft, autogenic bone marrow, or the combined use of autogenic

marrow with the other grafts.¹¹⁰ These grafts have the potential to facilitate direct osteogenesis, osteoinduction, and osteoconduction (not for marrow grafted alone) (See chapter 14 for more details).

B. BIOMATERIALS

Biomaterials, such as porous hydroxyapatite, tricalcium phosphate, bioactive glasses, or some biodegradable polymers are osteoinductive, meaning that they support the ingrowth of blood vessels, perivascular tissues, and osteoprogenitor cells from the recipient bed into the graft (or scaffold).¹¹⁰ Osteoconductive materials cannot induce bone formation at extraskeletal sites. Bone formation can be achieved by adding osteoinductive or osteogenic substances such as *in vitro* cultured osteoblasts (tissue engineering technique), autogenic marrow, GFs, or DBM (see Chapter 14).

C. LOCAL USE OF GROWTH PROMOTING SUBSTANCES

Growth factors (GFs) (peptide-signaling molecules) such as BMP, TGF- β , FGF, and PDGF have been identified at the fracture site and have been verified to have osteoinductive function by stimulating the generation of osteoprogenitor cells from undifferentiated perivascular mesenchymal cells and enhancing osteoblast proliferation and migration.^{110–112} The number of reports on animal models for *in vivo* effects of GFs on fracture healing has increased since several years ago, including the effect of local application of TGF- β on rat or rabbit tibial fracture,^{29,113} PDGF on rabbit tibial osteotomies,⁵¹ rhbFGF on rat fibular fracture,³³ and NGF- α (nerve growth factor) on rat rib fracture.¹¹⁴ GFs for clinical use will become commercially available in the near future.¹¹²

Besides its systemic effect on fracture healing (see below), prostaglandin E² (PGE²) has been demonstrated being released at the fracture site,¹¹⁵ which has been believed to function as a local mediator for fracture healing.^{110,116} Local infusion of PGE² in a rabbit fracture model has shown a stimulating effect on fracture healing (callus formation).²⁶

D. SYSTEMIC USE OF HORMONES OR DRUGS

Animal studies have indicated that growth hormone has stimulatory effects on fracture healing.^{79,80,117} Growth hormone has an initial stimulatory effect on external callus formation and also stimulates the haemopoietic system. Besides its local effect, PGE² also showed systemic stimulation of fracture healing in canine models.^{118,119} There are other systemic hormones or factors having potential effects of stimulating fracture healing, such as estrone,¹²⁰ 1 α -OH-D3,¹²¹ Factor XIII,¹²² or calcitonin.¹²³

A few drugs or agents have been found to have enhancing effects on fracture healing, which are diphenylhydantoin (Dilantin),¹²⁴ clodronate,²⁷ and vitamin D3.¹²⁵ Chinese herbal medicine has been used in Asian countries for fracture healing for more than two thousand years. However, only a few articles reporting animal studies on herbs have been published in the English literature.^{126,127}

E. BIOPHYSICAL STIMULATION

Mechanical stimulation, such as controlled micromotion, distraction, or weightbearing, has the ability of enhancing fracture healing,¹¹⁰ while early full weightbearing delays fracture healing.¹²⁸ The commonly used animal models for testing the effects of mechanical stimulation or weightbearing include fractures or osteotomies of rat femur,¹⁰ sheep tibia,¹²⁹ rabbit fibulae,¹³⁰ and canine tibia.⁶² Clinical trials showed that mechanical stimulation appears to enhance fracture healing applied through an external fixator.¹³¹ Encouraging clinical results on enhanced fracture healing by weight bearing have also been reported.¹³²

Electrical stimulation was a hot topic in the late 1970s and 1980s. Although only a small number of articles have been published in recent years, the validity of electrical stimulation for the treatment of delayed union and nonunion has been established. Generally, three types of electrical stimulation devices have been used, including constant direct current stimulation, electromagnetic stimulation,

and capacitive coupling. The commonly used animal models for testing the effects of electrical stimulation include delayed unions of rabbit fibulae⁹⁵ or radius,⁹³ nonunions of canine ulna,¹³³ fresh fracture of rabbit fibulae⁵² or tibia,¹³⁴ and the long bone lengthening model of canine tibia.¹³⁵

Duarte was the first to introduce the use of ultrasound for stimulating the growth of bone and fracture healing in fibular bone defects in the rabbit.¹³⁶ His results were further validated by others using the rabbit fibular model¹³⁷ and rat femoral model.¹³⁸ In a rabbit fibular model, it was shown that ultrasound stimulated fracture healing and the endogenous PGE² level was also significantly elevated, paralleling the enhanced bone healing,¹³⁹ leading to the suggestion that bone healing stimulated by ultrasound may be mediated via the production of PGE². Ultrasound also accelerates the clinical and overall healing of human fractures.¹⁴⁰

The mechanisms of enhancing fracture healing by physical stimulation methods may be that the physical signals trigger the activation or release of biochemical mediators for osteogenesis, such as GFs and PGE².¹¹⁶

VIII. OTHER FACTORS AFFECTING FRACTURE HEALING

A. EFFECTS OF FIXATION DEVICES

Stability of the fracture site is the most important factor in the successful treatment of fractures. Generally, a rigid, stable fixation allows early primary healing through the formation of internal callus, a stable but less rigid fixation leads to a slower secondary healing through the formation of external callus, and an unstable fixation tends to fail leading to delayed union or nonunion.^{116,141–143}

Controversy remains as to what kind of rigidity is the best for fracture healing. Rigid plate fixation (compression plating) stabilizes the fracture, allowing primary healing and early bone remodeling. However, rigid fixation minimizes granulation tissue and external callus, which may be caused by retardation of the release of GFs and PGE² at the fracture surfaces. Rigid fixation provides load shielding through the metal plate, causing lower bone strength. Less rigid plating creates slower secondary fracture healing and remodeling, causing less load shielding and resulting in higher ultimate strength of the bone union. The rigidity of external fixators has effects similar to plating, allowing primary healing with rigid fixation or secondary healing with less rigid or flexible fixation.

Intramedullary nailing is a successful procedure which allows some motion and loading at the bone ends (a less rigid fixation) and is usually associated with external callus formation. Intramedullary reaming causes circulatory disturbances in the inner 2/3 of the cortex, but it does not impede the formation of external callus and the damaged parts will be revascularized.

Based on the existing knowledge and authors' opinion, the future direction of research is to define a carefully selected range of rigidity which is rigid enough for a stable fixation and primary healing and also has certain flexibility for strong bone union by partial secondary healing. For testing fixation devices, larger animals such as rabbits,^{35,37,40,42–44} dogs,^{55,57,58,62} or sheep^{67,68} should be used (Table 1) due to the need for good size bones, better comparison to human conditions, and the convenience of implant manufacture.

B. EFFECTS OF DRUGS OR MEDICATION

Nonsteroidal antiinflammatory drugs (NSAIDs), especially indomethacin, are notorious substances for their inhibition effect on bone growth and fracture healing.^{144–146} Other NSAIDs having inhibiting effects on fracture healing include aspirin¹⁴⁵ and ibuprofen¹³

Several other agents or medications having inhibiting or discouraging effects on fracture healing have been demonstrated, such as estrogen,¹⁴⁷ cortisone,¹⁹ and irradiation.¹⁴⁸ There are also other drugs or substances for systemic use, such as ethane-1-hydroxy-1,1-diphosphonate (EHDP)⁶⁴ or alendronate,⁶⁶ which have been indicated to have no significant inhibiting effect on experimental fracture healing. Rat fracture models are ideal for testing the effect of systemic drugs on fracture healing.

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12 Animal Models for Testing Bioabsorbable Materials

Yuehuei H. An and Richard J. Friedman

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I. INTRODUCTION

Bioabsorbable materials have been studied in many aspects of orthopaedic surgery, including fixation of fractures, bone replacement, cartilage repair, meniscal repair, fixation of ligament, and drug delivery. However, the only major clinical application is fixation of fractures or osteotomies. Absorbable materials have been used in the form of screws, pins, and plates for orthopaedic, oral, and craniofacial surgery. Their mechanical properties can be altered to provide sufficient rigidity to allow bone healing, retain their mechanical properties for a period of time and then begin to undergo degradation. The most commonly used absorbable materials include polyglycolic acid (PGA), polylactic acid (PLA) and their copolymers.¹ Since the beginning of the concept of bioabsorbable materials by Kulkarni et al. in the 1960s,^{2,3} animal models have contributed tremendously to the success of the clinical use of the materials.

II. BASICS OF BIOABSORBABLE MATERIALS

A. COMMON BIOABSORBABLE MATERIALS

Polymers with the greatest potential for medical applications are polyhydroxyacids (polyesters). There are only few commercially available polyhydroxyacids, including polylactides, polyglycolide, poly(glycolide-co-lactide) (PGA-PLA), poly(glycolide-co-trimethylene carbonate), poly(p-dioxanone) (PDS), and polyhydroxybutyrate/valerate.

B. BIODEGRADATION

PGA, PLA, and their copolymers degrade in tissues by nonspecific hydrolytic scission of their ester bonds. By hydrolysis, PLA are changed to lactic acid which enters the tricarboxylic acid cycle and excreted in the forms of water and carbon dioxide. It is known that PGA also is broken down by enzymes like esterase.⁴

The biodegradation rate depends on many factors,⁵ such as the size of the implant, the kind of materials, the molecular weight of the material, the material phase (crystalline or amorphous), the presence of additives or impurities, the implantation sites (SC tissue or bone), the mechanism of hydrolysis (enzymes vs. water), and even the age⁶ of the animals.

Generally, pure PGA resorbs rapidly. As a result, either too much of a polymer load is produced or it is released too rapidly for it to be absorbed and excreted. Polyglycolic implants have produced fluid filled sterile sinuses with subsequent drainage.¹⁷ Pure PLA is highly crystalline and resorbs slowly. The amorphous state resorbs over 2–3 years, leaving behind crystallites that can elicit an inflammatory response. A PGA/PLA copolymer resorbs differently depending on the copolymer ratios.^{8,9}

C. MECHANICAL PROPERTIES OF BIOABSORBABLE MATERIALS

The requirement for polymer screws is that they have to be strong enough to hold the fracture fragments together until the fracture heals, which is normally 4–8 weeks. Most of the polymer screws or rods have been applied in areas of low stress.¹⁰ Polymer screws were not recommended for use without external support in places of high mechanical stress.^{10,11} Displacement of bone fragments has been reported, ranging from minor (several millimeters) to severe.^{12–14} Self-reinforced PGA (SR-PGA) has been reported to function better.^{15–18}

The tensile yield strength has been reported as 11 MPa to 72 MPa for PLA and 45 MPa for PGA.¹⁹ Flexural strengths for PLA are reported between 45 and 145 MPa. The highest flexural strengths are reported for PGA-fiber-reinforced PGA-composites, between 195 and 375 MPa. PGA reinforced by PLA fibers reaches a strength of 250 MPa. Although fiber reinforcement can improve the maximum strength of the material, it does not always improve the Young's modulus of the material, which remains very low (flexural modulus: 2 to 27 GPa for polymers compared to 200 GPa for stainless steel).

III. COMMONLY USED ANIMAL MODELS

A. BIOCOMPATIBILITY AND BIODEGRADATION TEST IN SOFT TISSUES

Subcutaneous or intramuscular implantation is the first *in vivo* step of testing a bioabsorbable material for its biocompatibility and degradation (Table 1). The rat is suggested as the first choice for soft tissue degradation studies because of its low cost and the rich background data available. In the rat, bone elongation ceases by age 6–9 months and 12–15 months of life remain after that. If the observation period is more than 15 months, rabbits should be used instead.

TABLE 1
Degradation of Bioabsorbable Materials *In Vivo* — Subcutaneous or Intramuscular Implantation

Animal	First author, year ^{Ref.}	Implant site	Material tested	Period (Wks)	Mass/mol. weight (MW) degradation	Inflammation reaction	Mechanical strength after implantation
Mouse	Gogolewski 1993 ²⁰	SC*	PLA, PHB†, PHB/VA§	24	56–99%/24 wks.	+ or ++	N/A
Rat	Pistner 1993 ²¹	IM‡	PLA (crystalline, 429,000 Mvis)	116	No change	+ at first few weeks	N/A
			PLA (amorphous, 203,000 Mvis)	116	100%	+++	N/A
			PLA (amorphous, 120,000 Mvis)	116	100%	++	N/A
	Gerlach 1993 ²²	IM	PLA rods	108	N/A	+	50% of original at 4 wks.
	Bos 1991 ²³	SC	PLA	143	14%/80 wks.	+ or ++	N/A
	Schakenraad 1989 ²⁴	SC	Glycine/DL-lactic acid discs	10	100%	+++	N/A
Rabbit	Nakamura 1989 ²⁵	IM	PLA, purified	52	No change/40 wks.	N/A	50% decrease
	Richards 1991 ²⁶	IM	4 Poly (phosphoesters)	70	80%/70 wks.	+	N/A
	Tormälä 1991 ¹⁸	SC	PGA	8	N/A	N/A	Lost mech. strength at 4–7 wks.
	Matsuse 1992 ²⁷	SC	PLA rods	78	91% (MW)/12 wks.	+	100% decrease at 25 wks.
	Kumta 1992 ²⁸	SC	PGA rods	21	N/A	N/A	64% decrease at 2 wks.
	Tschakaloff 1994 ²⁹	SC	PLA plates	6	87% (MW)	No	N/A
	Bhatia 1994 ⁶	SC, IM, bone	Absorbable pins (Orthosorb)	5	N/A	N/A	Decreased differently at each site
	Matsuse 1995 ³⁰	SC	PLA rods	276	100%/276 wks.	No	100% decrease at 276 wks.
	Ertel 1995 ³¹	IM, bone	Poly(DTH carbonate) and PDS pins	26	Started at 26 wks. PDS partially absorbed	No ++, bone resorption	N/A N/A

* SC = subcutaneous

‡ IM = intramuscular

† PHB = poly (3-hydroxybutyrate)

§ PHB/VA = poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

+ = slight

++ = moderate

+++ = significant.

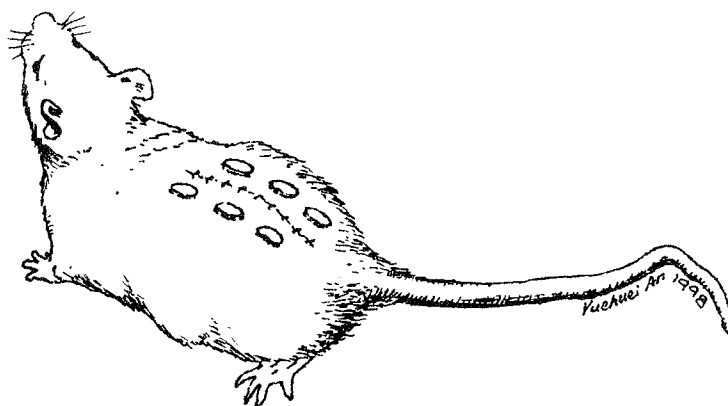


FIGURE 1. Schematic diagram showing different soft tissue implantations. Up to six disk implants can be inserted subcutaneously or intramuscularly on the back of a rat.

Implants are inserted into pouches in SC tissue or in muscles. It makes no difference where in the SC tissue (the back, inner aspect of the thighs, or abdominal wall) or in which muscle (the back muscles, thigh muscles, or abdominal muscles) the implant is placed. However, most researchers implant into the back SC tissue or the back muscles, simply because of the convenience and the large area or volume of the tissues.

Up to six implants (discs, 10 mm diam. and 1 mm thickness) could be implanted on the back of each rat, while 8–10 implants could be inserted into the back of each rabbit (Figure 1). Implantations can be done through one large midline incision or multiple small ones. The key is that the sample is placed in the right layer, either in the loose SC tissue space or in the paravertebral muscles. For testing mechanical degradation, rod-shaped samples (3.2 mm diam. and 50 mm length) are often used. At least six rods could be implanted into the back of an adult rabbit. The time periods used for the observation of degradation range from one month to five yrs. For more than 1.5 yrs' observation, rabbits should be used because the adult life of rats is less than 1.5 yr.

Standardizing sample shape and size is very important for comparing data between research groups. Soft tissue implantation is suitable for standardized testing of mechanical and mass degradation because the implant is more retrievable compared to in-bone implantations. An absorbable material sample measuring 3.2 mm in diameter and 50 mm in length is suggested for studying the mechanical degradation of materials, which has been used by many investigators.^{18,22,27,28,30} A disc measuring 10 mm in diameter and 1 mm in thickness may be used for mass degradation studies using histological method or molecular weight degradation studies using a viscosimeter.

B. BIODEGRADATION IN BONE TISSUE

Bioabsorbable implant degradation in bone tissues are the further step after soft tissue implantation, when the material is to be used inside of or in contact with bone (Table 2). These models test the biocompatibility and degradation pattern of the material. Although different animals have been used, the rabbit is the most popular one.

Implants in the forms of rods, plugs, pins, or screws are most often implanted in the cancellous bone of the distal femur in the rabbit through drill holes (Figure 2A,B). Diaphyseal implantation has also been used in different animals (Figure 2C).

TABLE 2
Animal Models of Bioabsorbable Implant Degradation in Bone Tissues

Animal	Bone	First author, year ^{Ref.}	Bone defect	Material tested	Period (wks.)	Degradation	Inflammation reaction	Mechanical properties of materials
Rat	Femur	Curright 1974 ⁸	Upper femur drill hole	PGA, PLA, or copolymers	31	100% in 14–31 wks.	No	N/A
Rabbit	Femur	Väinönpää 1986 ³²	Intercondylar area	PGA rods	12	Started in 6 wks.	No	N/A
		Mäkelä 1989 ³³	Intercondylar area	PDS rods	36	Nearly 100% in 36 wks.	N/A	N/A
		Matsusue 1992 ²⁷	Intercondylar area	PLA rods	70	22%/52 wks., 70%/78 wks.	+	100% loss at 25 wks.
		Matsusue 1995 ³⁰	Intercondylar area	PLA rods	248	100%/24 in 8 wks.	No	N/A
		Kumta 1992 ²⁸	Medullary canal	PGA rods	3	Broken down in 3 wks.	N/A	73% Bending str.
		Böstman 1994 ³⁴	Intercondylar area	PGA pins and screws	48	100% in 36 wks.	?	N/A
		Fini 1995 ³⁵	Medullary canal	PLA (high MW) rods	64	0%	No	Flexional stiffness
		Knowles 1992 ³⁶	Diaphyseal drill holes	Polyhydroxybutyrate plugs	19	—	N/A	N/A for the material
Tibia		Väinönpää 1986 ³⁷	Diaphyseal drill holes	PGA rods	12	Started in 6 wks.	No	N/A
		Ertel 1995 ³¹	Medial tibial plateau	Poly(DTH carbonate) and PDS pins	26	Started at 26 wks.	No	N/A
Peri-orbital						PDS partially absorbed	++ osteolysis	N/A
Dog	Femur	Kellman 1994 ³⁸	1.5 mm drill holes	PLA, PLATMC*, PGA screws	32	N/A	++	No strength at 32 wks.
		Miettinen 1992 ³⁹	Intramedullary implant	SR-PGA implant	24	Started in 3–6 wks.	No	N/A
Goat	Femur	Suganuma 1993 ⁴⁰	Distal metaphysis	PLA in a bone chamber	24	20% in 24 wks.	++	N/A
Pig	Mandible	Verheyen 1993 ⁴¹	Diaphysis drill holes	PLA, PLA/HA plugs	104	Obvious for pure PLA	Lymph nodes	N/A
		Schliephake 1993 ⁴²	Hole below the mandibular canal	Polydioxanone pins	26	100% in 22–26 wks.	N/A	N/A

* PLA/TMC = polylactic acid/trimethylene carbonate copolymer
+ = slight; ++ = moderate.

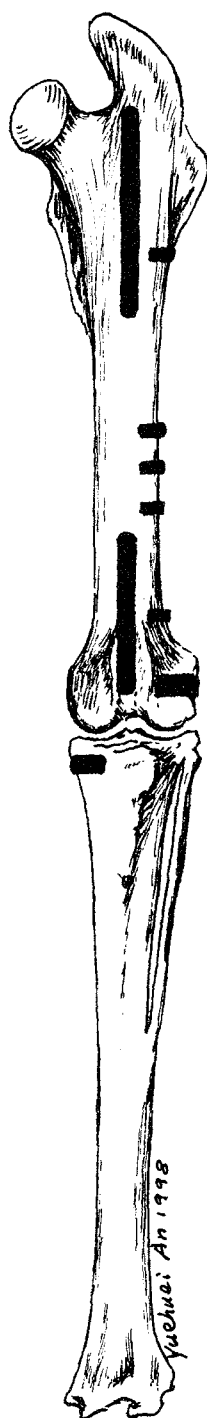


FIGURE 2. Schematic diagrams showing different in-bone implantations of bioabsorbable materials.

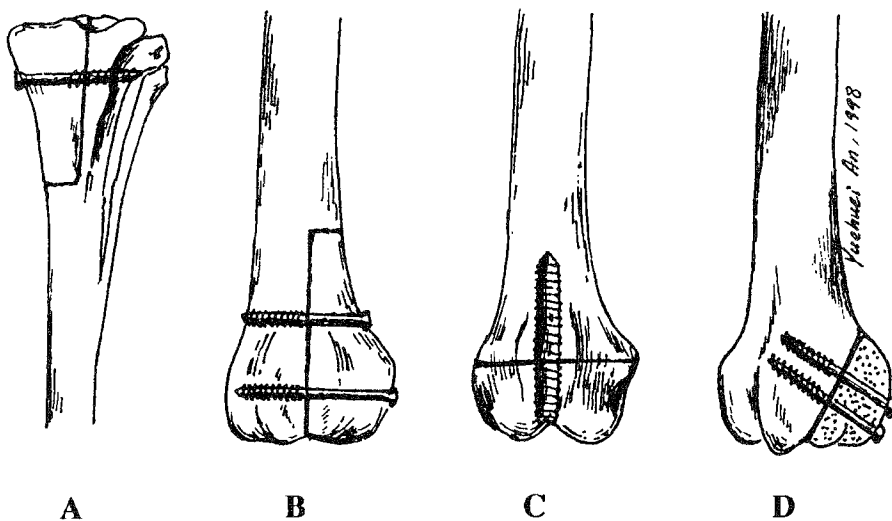


FIGURE 3. Schematic diagrams showing different epiphysometaphyseal implantation models: (A) osteotomy of medial tibial plateau in the rabbit; (B) Salter-Harris type IV fracture in the dog; (C) the femoral transcondylar osteotomy in the rabbit; and (D) the lateral femoral condyle osteotomy in the dog.

C. FIXATION OF FRACTURE AND OSTEOTOMY

1. Epiphysometaphyseal Osteotomy Models

The representative models of epiphysometaphyseal osteotomies (Table 3) include the osteotomy of medial tibial plateau in the rabbit (Figure 3A),⁵² Salter-Harris type IV fracture in the dog (Figure 3B),⁵⁸ the femoral transcondylar osteotomy in the rabbit (Figure 3C),^{37,50} and the lateral femoral condyle osteotomy in the dog (Figure 3D).⁵³

Femoral transcondylar osteotomy in rabbits is a well-established model.^{37,50} Salter-Harris type IV fracture in the dog or the similar osteotomy created at the medial tibial plateau by Matsusue et al.⁵² is another excellent model in the epiphysometaphyseal area. The forces that the absorbable screw is subjected to is simple, either on the lateral or the medial side of the knee joint. The proximal-to-distal shearing force is sustained by the transverse portion of the osteotomy. Another interesting model is the subcapital femoral neck osteotomy in sheep, which is more demanding for fixation because of weight-bearing.⁵⁵

For the lateral femoral condyle osteotomy in dogs (from the authors' laboratory), an unilateral model is recommended because of the amount of surgical injury.⁵³ Through a lateral approach, an osteotomy from the superolateral aspect of the condyle to the intercondylar notch in the knee joint was made and the osteotomy was secured with two screws. The advantage of this model is the instability of the osteotomy (high shear force to the screws), which challenges the efficacy of the fixation devices. The effect of bioabsorbable screw on the cancellous bone healing can be evaluated with a trephined plug model in canine distal femur (Figure 4).⁵³

2. Diaphyseal Fracture Models

Very few diaphyseal models are available (Table 4). The first diaphyseal model was reported by Vainionpää et al.,⁵⁹ in which a tibial osteotomy was fixed with a T-shaped PGA/PLA copolymer implant (Figure 5A). Oblique fractures of metacarpal bones in calves were also fixed with PLA

TABLE 3
Animal Models of Fixation of Osteotomies of Epiphysometaphyseal Area of Long Bones and Irregular Bones of the Limbs
Using Bioabsorbable Materials

Animal	Bone	First author, year ^{Ref.}	Osteotomy or fracture	Fixation method	Period (Wks.)	Degradation	Inflammation reaction	Fracture healing
Rat	Femur	Majola 1991 ⁴³	Transcondylar	SR-PLA, SR-PDLA/PLLA	48	Started in 12 wks.	No	95% healed at 48 wks.
Rabbit	Femur	Vainionpää 1986 ³⁷	Transcondylar	PGA thread and rods	24	Started at six wks.	N/A	Healed at six wks.
		Vihonen 1987 ⁴⁴	Transcondylar	PGA thread (Dexon)	24	N/A	N/A	79% healed at six wks.
		Vihonen 1988 ⁴⁵	Transcondylar	Bone cement, PGA thread	48	N/A	N/A	65% healed
		Vasenius 1990 ⁴⁶	Transcondylar	SR-PGA rods	48	100% in 24–36 wks.	No	99% healed
		Plaga 1992 ⁴⁷	Oblique medial condyle	PDS pins	6	N/A	N/A	86% healed
		Böstman 1992 ⁴⁸	Transcondylar	PGA screws	36	100%/36 wks.	++	Healed
		Päiväranta 1993 ⁴⁹	Transcondylar	PGA screws	48	100% /36 wks.	+ or ++	Healed
				PLA screws	48	0% /48 wks.	+ or ++	Healed
		Pihlajamäki 1994 ⁵⁰	Transcondylar	PLA expansion plug	24	N/A	No	Healed
		Pihlajamäki 1994 ⁵¹	Transcondylar	SR-PLA expansion plug	24	N/A	No	75% healed at 24 wks.
Dog	Tibia	Matsue 1991 ⁵²	Proximal tibial osteotomy	PLA or stainless steel screws	16	N/A	+	Healed
	Femur	An 1997 ⁵³	Lateral condyle osteotomy	PGA/PLA screws	68	>95% 68 wks.	No	Healed/8 wks.
	Total hip	Orsuka 1994 ⁵⁴	Fixing acetabular component	PLA screws	14	N/A	N/A	Healed OK
Sheep	Femur	Jukkala-Partio 1997 ⁵⁵	Subcapital osteotomy	SR-PLA lag-screws	12	N/A	?	86% healed
	Tibia	Weiler 1996 ⁵⁶	Medial condyle	PGA rods (Biofix)	12	N/A	+++	Healed
	Ulnae	Manninen 1992 ⁵⁷	Transverse olecranon	PLA screws	12	N/A	N/A	Healed
Goat	Femur	Donigian 1993 ¹³	Salter-Harris IV fracture	PLA, polydioxanone pins	8	N/A	No	Healed

*PHBA = poly-β-hydroxybutyric acid
+ = slight
++ = moderate
+++ = significant

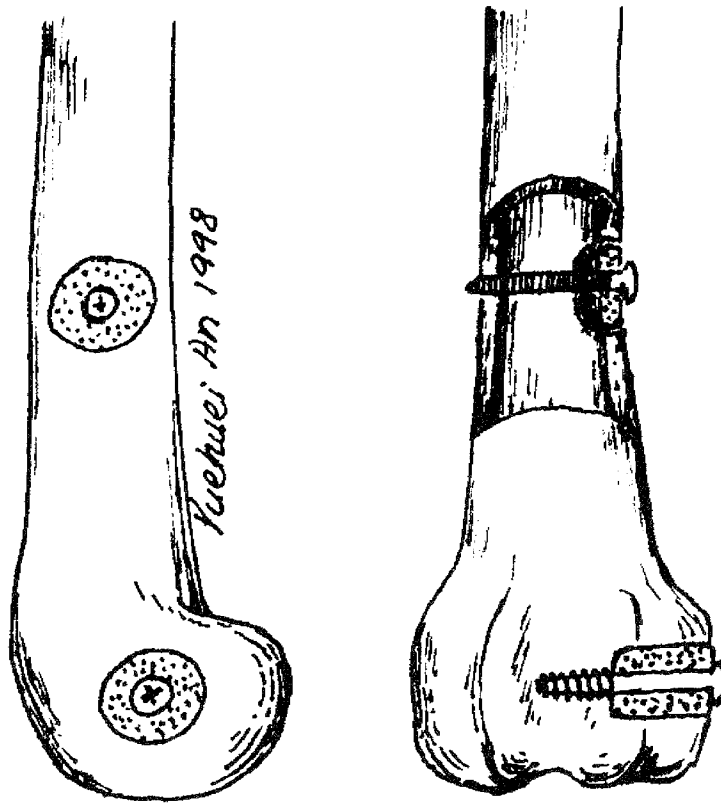


FIGURE 4. Schematic diagrams showing trephine osteotomies made at both diaphyseal and epiphysometaphyseal areas in the dog.

tension screws (Figure 5 B).⁶¹ Models of femoral osteotomy fixed with PLA, SR-PLA, or SR-PGA intramedullary rods have been reported in the dog, rabbit, and cat (Figure 5C,D).^{57,58,60} The intramedullary rod can be inserted through the greater trochanter or the intercondylar notch. The studies showed that the bioabsorbable rods were strong enough to be used in intramedullary nailing of femoral diaphyseal osteotomies in the three animal species. In the authors' laboratory, healing of a trephined femoral diaphyseal osteotomy in the dog has been used to observe the effect of fixation using a PGA/PLA (80/20 ratio) screw (Figure 4).⁵³ In most of the reports, bone consolidation normally occurred in 6–8 weeks.

3. Maxillofacial Bone Fracture Models

Models of mandibular osteotomies are the most popular and well established in dogs and sheep (Figure 6A). Rabbits are not suitable for mandibular models because they do not have a long mandible for fixation instrumentation. Very few models using zygomatic or nasal bones have been reported using rabbits (Figure 6B,C).^{29,62} Based on anatomical features, dogs can also be used for these two models. The most common form of bioabsorbable fixation devices for these models are

TABLE 4
Animal Models of Fixation of Diaphyseal Osteotomy Using Bioabsorbable Materials

Animal	Bone	First author, year ^{ref.}	Osteotomy or fracture	Fixation method	Period (Wks.)	Degradation	Inflammation reaction	Fracture healing
Rabbit	Tibia	Vainionpää 1986 ⁵⁹	T	Carbon fiber-reinforced PGA/PLA and PHBA* implant	24	Started at six wks.	N/A	Healed at six wks.
Dog	Femur	Manninen 1993 ⁵⁷	T	SR-PLA intramedullary rods	48	N/A	No	Healed
	Femur	Miettinen 1992 ⁶⁰	T	SR-PLA or SR-PGA	48	Disappeared @ 24 wks.	No	Healed/6 wks.
		An 1997 ⁵³	Trephined	PGA/PLA screws	68	>95% 68 wks.	No	Healed/8 wks.
Cat	Femur	Hara 1994 ⁵⁸	T	PLA intramedullary rods	16	N/A	No	Healed/large callus
Calf	Metacarpal	Illi 1992 ⁶¹	45°O	PLA or AO metallic screws	6	0% or N/A	No	Healed

* PHBA = poly-β-hydroxybutyric acid

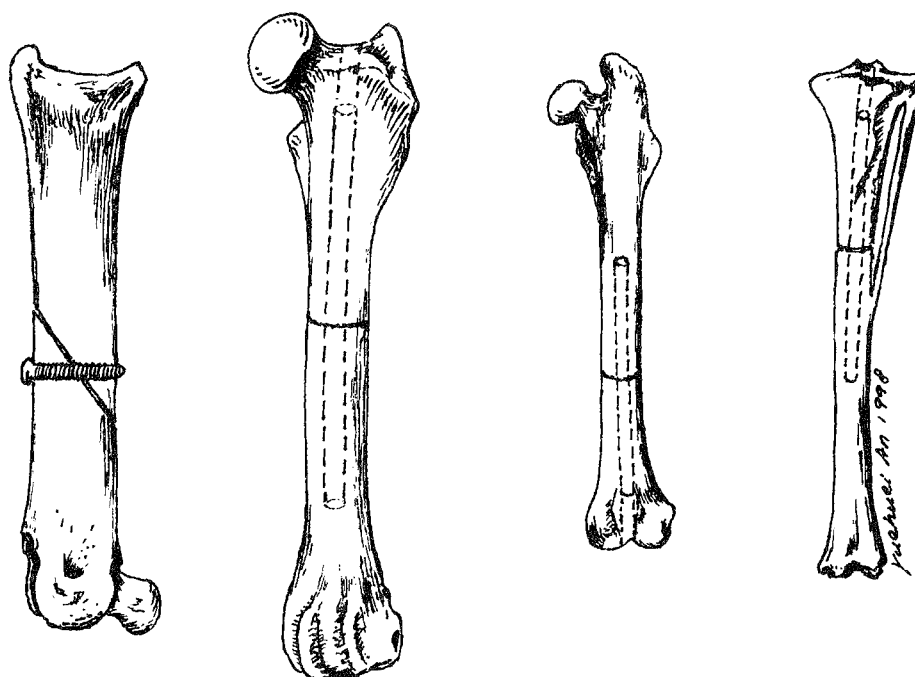


FIGURE 5. Schematic diagrams showing different diaphyseal fracture models: (A) an oblique fracture of metacarpal bone in calves fixed polymer screws (lateral view);⁶¹ (B) a femoral osteotomy fixed with a polymer intramedullary rod inserted through greater trochanter in the dog;⁶⁰ (C) a femoral osteotomy fixed with a polymer intramedullary rod inserted through intercondylar notch in the rabbit;⁵⁷ and (D) a potential fracture fixation model using an intramedullary rod in the rabbit.

mini plate and screws although the use of absorbable suture and screw only have been reported (Table 5).

D. BONE REPLACEMENT

In the case of repairing a bone defect, bioabsorbable materials are often used independently for bone conduction,^{69,70} or as a part of the substitute (with HA^{71,72} or proteolipid⁷³), or as a carrier for GFs,^{74,75} BMP,^{76–79} and other bone elements (Table 6).^{84–87} The other function of absorbable materials may be no more than a scaffolding for bone conduction^{69,70} or guided bone regeneration.⁸²

E. DRUG DELIVERY

Typical bioabsorbable drug delivery systems are designed to introduce antibiotics for local use (Table 6). PLA and oligomer dideoxykanamycin B were used as a delivery system to treat osteomyelitis in the rabbit femur.¹⁰¹ Garvin et al.¹⁰³ reported the use of PLA/PGA gentamicin implant in the treatment of osteomyelitis in a canine model. Overbeck et al.¹⁰⁴ implanted a novel ciprofloxacin-containing PGA cylinder into the medullary canal of the proximal femur in the rabbit and showed that a significant greater concentration of ciprofloxacin into bone than has been reported for gentamicin cement beads. Langer reviewed drug delivery systems made from bioabsorbable materials.¹¹¹

Degradable polymers can also be used for the controlled release of insulin, GFs,^{74,78} indomethacin,¹¹² and angiogenesis inhibitors.

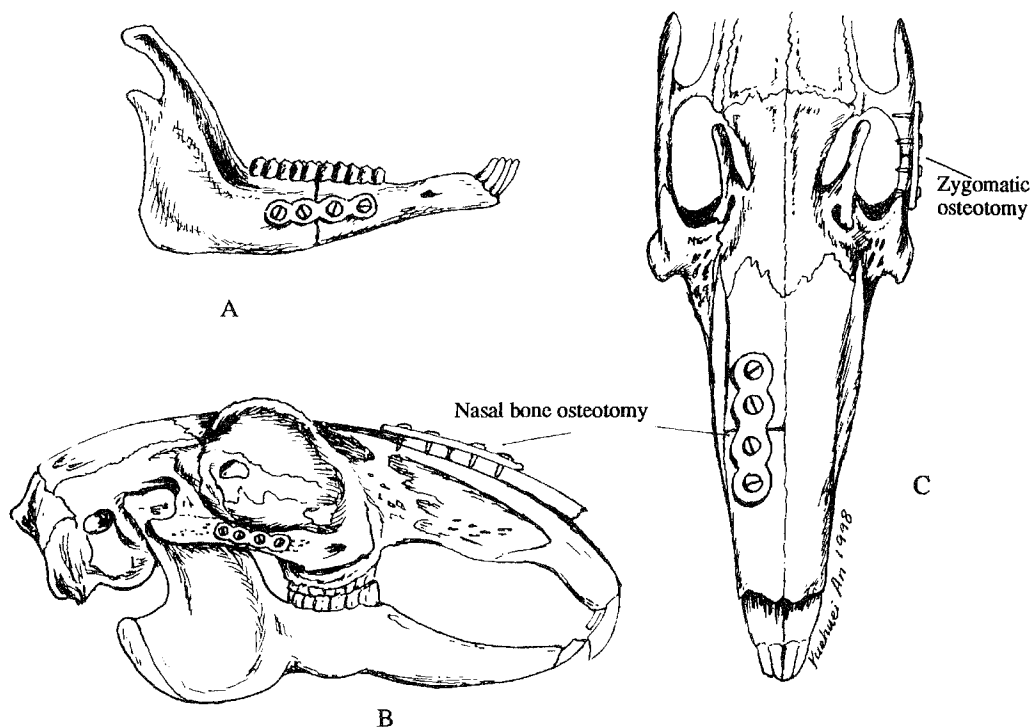


FIGURE 6. Schematic diagrams showing maxillofacial bone osteotomy and fixation with bioabsorbable plate and screws: (A) a mandibular osteotomy in the goat, (B) and (C) zygomatic bone and nasal bone osteotomies in the rabbit.

F. REPAIR OF CARTILAGE DEFECT

With variable results, the typical studies of repairing cartilage defects using bioabsorbable materials include the use of PGA rods,³⁴ PGA or PLA combined with perichondrium in the rabbit^{88,113} and PLA/PGA implants with impregnated DBM powder¹¹⁴ or TGF- β ¹¹⁵ (Table 6).

Seeding cells onto porous PLA polymers is a new approach. Freed et al. studied neocartilage formation *in vitro* and *in vivo* using porous PGA and PLA scaffolds.¹¹⁶ They also implanted chondrocyte-seeded absorbable implant to repair cartilage defects with promising results.⁸⁹ In recent years, more investigations have been reported on the repair of cartilage defect using cell-seeded absorbable implants.^{90,91} There is no doubt that the use of tissue engineering principles for cartilage repair is the major direction of research.

G. REPAIR OF MENISCUS

A porous PLA scaffold was used by Klompmaker et al. to repair meniscal lesions.⁹² It was found that the biodegradable implant with a porous structure (size 100–300 μm) could provide guidance for vascular ingrowth into the defect (Table 6). Recently, the same group reconstructed canine meniscus using a porous copoly(L-lactide/ ϵ -caprolactone) material and found the gap formation between implant sides and meniscal tissue could be prevented, but the adherence of implant to the underlying meniscal tissue seemed to be a problem.⁹³ More recently, a new PGA/PLA staple has been tested for meniscal repair.⁹⁴ The results showed that the staple provided greater tensile strength than PDS sutures to the repaired meniscus.

TABLE 5
Animal Models of Fixation of Osteotomies of Maxillofacial Bones Using Bioabsorbable Materials

Animal	Bone	First author, year ^{ref.}	Osteotomy or fracture	Fixation method	Period (Wks.)	Degradation reaction	Inflammation healing	Fracture
Rabbit	Zygomatic	Thaller 1993 ⁶²	Saw osteotomy	PGA plate and screws	16	N/A	N/A	Healed
	Calvarial bone	Eppley 1995 ⁶³	Circular defect	PLA/PGA plate and screws	52	100%/52 wks.	No	Healed/24 wks.
	Nasal bone	Tschakaloff 1994 ²⁹	Transverse osteotomy	PLA plate and screws	6	75% mol. weight	No	N/A
	Mandible	Getter 1972 ⁶⁴	Transverse osteotomy	PLA plate and screws	32	100%/32 wks.	+	Healed
		Leenslag 1987 ⁶⁵	Transverse osteotomy	PLA plate and screws	11	Some at 11 wks.	No	Healed
Dog	Mandible	Gerlach 1993 ²²	Transverse osteotomy	PLA plate and screws	100	N/A	+	Healed/8 wks.
		Tamis 1995 ⁶⁶	Transverse osteotomy	PLA plate and screws	18	Not much/18 wks	No	Healed/12 wks.
		Hara 1994 ⁶⁷	Salter-Harris IV fracture	PLA screws	24	Started/24 wks.	No	Healed/4-8 wks.
		Leenslag 1987 ⁶⁵	Transverse osteotomy	PLA plate and screws	11	Some at 11 wks.	No	Healed
	Mandible	Bos 1989 ⁶⁸	Transverse osteotomy	PLA plate and screws	11	Not much/11 wks.	No	Healed
Sheep	Mandible	Suuronen 1991 ¹⁷	Neck of condylar process	SR-PLA screws	24	N/A	N/A	Healed

* PHBA = poly-β-hydroxybutyric acid; + = slight reaction.

TABLE 6
Applications of Bioabsorbable Materials in Animal Models and Human Subjects in Orthopaedic Research

Application	Subject	Location	Materials	First author, year ^{ref.}
Fixation of fracture or osteotomy Bone filler or substitute	Animals	Various	Various	(For references see Tables 3, 4, 5)
	Rat	Tibia and humerus drill holes	PLA/PGA	Hollinger 1983 ⁶⁹
	Rat	Femur, drill hole	PLA/HA composite	Higashi 1986 ⁷¹
	Dog	Mandibular discontinuities	PLA/PGA + proteolipid	Hollinger 1987 ⁷³
	Rabbit	Calvarial defect	PLA/PGA/HA	Antikainen 1992 ⁷²
	Rat	Critical size defect (5 mm) in mandible	PLA/PGA membranes	Sanberg 1993 ⁸⁰
	Rat	Femur/Around metaphyseal bone	SR-PGA membranes	Ashammakhi 1994 ⁸¹
	Rat	Femur/around bone surfaces	SR-PGA membranes	Ashammakhi 1995 ⁸²
	Rabbit	Augmenting distal femoral drill holes	SR-PGA membranes	Ashammakhi 1995 ⁷⁰
	Human	Femoral nonunion	h-BMP in PLA/PGA copolymer	Johnson 1988 ⁸³
	Rabbit	Calvarial defect	DFDB* in PLA/PGA	Schmitz 1988 ⁸⁴
	Rat	Osteogenesis in muscles	DBM† in polyorthoester	Pinholt 1991 ⁸⁵
	Rabbit	Calvarial defect	Osteoinductive protein in PLA/PGA	Turk 1993 ⁷⁵
	Dog	Osteogenesis in SC‡ tissue	PCBM§ in PLA mesh	Kinoshita 1993 ⁸⁶
Carriers for osteogenic substances	Rat	Osteogenesis in SC tissue	DBM in PLA	Saitoh 1994 ⁸⁷
	Rat	Calvarial defect	BMP in PLA disc	Miki 1994 ⁷⁶
	Rat	Calvarial defect	BMP in PLA/PGA microparticles	Kenley 1994 ⁷⁷
	Rat	Osteogenesis in calvarial defect	IGF-I in polyorthoester	Busch 1996 ⁷⁴
	Rat	Osteogenesis in SC tissue	BMP in PLA/PGA/blood clot	Alpaslan 1996 ⁷⁹
	Rat	Osteogenesis in muscles	BMP in PLG/PEG reservoirs	Yamazaki 1996 ⁷⁸
	Mouse			

Repair of cartilage defect	Rabbit	Defect in distal femoral joint	PLA/periosteum	von Schroeder 1991 ⁸⁸
	Rabbit	Defect in distal femoral joint	PGA scaffold + chondrocytes	Freed 1994 ⁸⁹
	Rabbit	Defect in distal femoral joint	PLA scaffold + chondrocytes	Vasanti 1994 ⁹⁰
	Rabbit	Defect on medial femoral condyle	Porous PLA + perichondrocytes	Chu 1995 ⁹¹
Repair of meniscus	Dog	Lateral menisci	Porous PLA implant	Klompmaier 1991 ⁹²
	Dog	Lateral menisci	Copoly(L-lactide/ε-caprolactone)	de Groot 1997 ⁹³
	Dog	Medial menisci peripheral third incision	PGA/PLA staples or 3-0 PDS suture	Koukoubis 1997 ⁹⁴
Repair or replacement of ligament and tendon	Dog/Rabbit/Dog	Patellar/Achilles/med. collateral lig.	PLA-carbon scaffolding ribbons	Parsons 1983 ⁹⁵
	Sheep	ACL reconstruction	Braided PLA augmentation	Laitinen 1993 ⁹⁶
	Sheep	PDS-augmented patellar tendon	PDS cords	Holzmüller 1994 ⁹⁷
	Rabbit	Achilles tendon laceration or defect	A PGA/Dacron device	Rodkey 1985 ⁹⁸
Fixation of ligament and tendon	Human	Arthroscopic patellar tendon graft	PLA interference screws	Barber 1995 ⁹⁹
	Human	Ulnar collateral ligament of the 1st metacarpophalangeal joint	SR-PLA mini tack fixation	Juutilainen 1996 ¹⁰⁰
Drug (antibiotics) delivery	Rabbit	Implanted into distal femur	PLA/Kanamycin	Wei 1991 ¹⁰¹
	Rabbit	Tibial fracture, infection	PLA/cefazolin microspheres	Jacob 1993 ¹⁰²
	Dog	Tibia osteomyelitis	PLA/PGA implant + gentamicin	Garvin 1994 ¹⁰³
	Rabbit	Inserted into proximal femur	PGA/Ciprofloxacin cylinder	Overbeck 1995 ¹⁰⁴
	Rabbit	Tibial osteomyelitis	PLA, PLA/PGA + vancomycin	Calhoun 1997 ¹⁰⁵
Conduits for nerve repair	Human	Digital nerve defect	PGA tube	Mackinnon 1990 ¹⁰⁶
	Primate	Median nerve defect	PGA tube	Hentz 1991 ¹⁰⁷
	Rat	Ischiatic nerve defect	PLA/6-caprolactone conduit	Nicoli Aldini 1996 ¹⁰⁸
Scaffold for vessel regeneration	Rat	Abdominal aorta graft	Microporous polyurethane/PLA	van der Lei 1987 ¹⁰⁹
	Rabbit	Infrarenal aorta	Woven PDS graft	Greisler 1991 ¹¹⁰

* DFDB = demineralized freeze-dried bone

† DBM = demineralized bone matrix

‡ SC = subcutaneous tissue

§ PCBM = particulate cancellous bone and marrow

¶ PLG/PEG = poly(lactide-co-glycolide)/poly(ethylene glycol)

We believe that with the rapid development of tissue engineering, cell seeded and/or growth factor-impregnated implant is the main direction of research.

H. REPAIR OF TENDONS AND LIGAMENTS

The replacement of tendons and ligaments with bioabsorbable materials is still in the early stage of research (Table 6). The concept was given by Parson et al. in 1983.⁹⁵ They reported the use of a PLA-filamentous carbon fibers to replace tendons and ligaments. Rodkey et al.⁹⁸ found that a PGA/Dacron material had adequate strength and physical properties to be used both for primary tenorrhaphy and bridging tendon defect in a rabbit Achilles tendon model. Recently, braided PLA and PDS cords have been used to augment fascia lata and patellar tendon respectively in sheep for ACL reconstruction with promising results.^{96,97}

Although no animal models have been reported, fixation of tendon and ligament to bone by an absorbable interference screw and a mini tack in human patients have been established recently.^{99,100} Again, we believe that cell seeded and/or growth factor-impregnated porous implant made from bioabsorbable materials for the replacement of tendons and ligaments will be one of the future directions of the research.

I. SMALL BLOOD VESSEL AND NERVE REGENERATION

Absorbable materials have been used for small vessel and nerve regeneration (Table 6). The typical animal studies of vessel regeneration using absorbable materials were reported by van der Lei et al.¹⁰⁹ Microporous, compliant, vascular grafts made from a mixture of polyurethane (95%) and PLA (5%) can function as a scaffold for the regeneration of small-caliber arteries.¹⁰⁹ Common models for studying small vessel graft include rat abdominal aorta, rabbit carotid artery and infrarenal aorta, and canine femoral, carotid artery, and aorta.

For nerve regeneration, several materials, such as PGA tube,^{106,107} PLA-co-6-caprolactone conduit,¹⁰⁸ and other forms of absorbable guides,¹¹⁷ have been investigated with controversial results. The common model for experimental nerve regeneration is ischiatic nerve defect in the rat.

IV. EVALUATION METHODS

Animals should be inspected daily for evidence of foreign-body reactions (sterile sepsis). A radiograph is used for determining the location of an osteotomy, the quality of the fixation, the existence of osteolysis, and the progress of bone healing (see Chapters 11 and 13).

Histological analysis is used to evaluate bone healing (see Chapters 11 and 13), tissue response to bioabsorbable devices, and the mass degradation of the polymer. Ground sectioning can be chosen for its advantage that the residues of polymer may be preserved during specimen processing and suitability for specimens containing metal devices.

Bending and tensile tests are commonly used to testing the strength of absorbable devices before and after implantation. A screw pullout test also has been used. An indentation test can be used to test the strength of bone around a bioabsorbable screw or rod.⁵³ The strength of osteotomy sites of diaphysis can be tested to failure using a torsional test or bending test.

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13 Animal Models of Bone Defect Repair

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I. INTRODUCTION

The history of bone grafts for repairing defects can be traced back a few hundred years. Although the work was started at a time that lacked basic science and technology, the efforts led to the establishment of bone grafting techniques by the early twentieth century. Due to the complicated nature, bone defects caused by various conditions have been challenging orthopaedic surgeons and related biomedical scientists for some time, and are continuing to inspire them to seek better alternatives and new solutions. To achieve their goals, animal models of bone defects for bone grafting are essential.

TABLE 1
Selected Heterotopic Models of Osteogenesis

Animal	Implant site	Material tested	First author, year ^{Ref.}
Nude mouse	SC*	Human osteoblasts, diffusion chamber	Gotoh 1995 ¹
	IP	Human osteoprogenitor cells in diffusion chamber	Gundle 1995 ²
	SC	Osteosarcoma cells	Hara 1996 ³
Syngenic mouse	IP‡	Rodent Achilles tendons in diffusion chamber	Gooney 1993 ⁴
Nude rat	IMO	Rat and human DBM	Aspenberg 1989 ⁵
Rat	SC	Inductive bovine DBM	Nathan 1988 ⁶
	SC, IM	Porous ceramics + marrow cells	Ohgushi 1989 ⁷
	IM	Fibroblast growth factor	Aspenberg 1989 ⁸
	SC	DBM powder	Mohr 1991 ⁹
	SC	Alkaline phosphatase	Beertsen 1992 ¹⁰
	IM	Rabbit BMP in diffusion chamber	Ono 1994 ¹¹
	IM	Multiple bone substitutes (6 each)	Begley 1995 ¹²
	SC, IM	Porous HA/TCP	Yang 1996 ¹³
	MES§	Marrow stromal osteoblast in PLA/PGA foam	Ishaug-Riley ¹⁴
	IP	Rabbit marrow cells in diffusion chamber	Ashton 1980 ¹⁵
Rabbit	IM	Bone marrow, DBM, collagen in diffusion chamber	Strates 1989 ¹⁶
	IM	Composite of HA and periosteum	Kurashina 1995 ¹⁷
	IM	Porous HA	Ripamonti 1996 ¹⁸
	SC, IM	Porous HA/TCP	Yang 1996 ¹³
	IM	Porous HA	Ripamonti 1996 ¹⁸
Dog	SC, IM	Porous HA/TCP	Yang 1996 ¹³
	SC, IM	Porous HA/TCP	Yang 1996 ¹³
Pig	SC, IM	Porous HA/TCP	Yang 1996 ¹³
Goat	SC, IM	Porous HA/TCP	Yang 1996 ¹³
Primate	IM	Allogenic DBM and porous HA	el Deeb 1989 ¹⁹
	IM	Porous HA	Ripamonti 1996 ¹⁸

* SC = Subcutaneous tissue

† IM = Intramuscular

‡ IP = Intraperitoneal

§ MES = Mesentery

II. ANIMAL MODELS FOR BONE DEFECT REPAIR

A. HETEROTOPIC MODELS OF OSTEOGENESIS

There are several major heterotopic models for testing *in vivo* osteogenesis, the subcutaneous model, the intramuscular model, the intraperitoneal model, and the mesentery model, which are often used as the first step before a bone defect model (Table 1).²⁰ Substances, constructs, or diffusion chamber containing osteogenic materials were implanted in the three anatomical sites in mice, rats, rabbits, dogs, pigs, goats, or primates. After 3–24 weeks the implants were explanted and studied radiographically and histologically to identify the existence and extent of new bone. The effects of animal species on the amount of bone formation have been demonstrated.¹³ There are also effects of implantation sites and local environment on the amount or extent of bone formation.^{20,21}

The diffusion chamber is made from a plastic ring (2 mm-thick, 9 mm diam.) bounded by two porous cellulose acetate and nitrate membranes (100 µm thickness, 0.45 µm pore size) and a chamber volume about 130 µl (Millipore Corporation, Sedford, MA).^{2,4,11,15} For example, a diffusion chamber

containing rabbit BMP was implanted in the abdominal muscle of the rat. After implantation, cartilage differentiated around the chamber in 1–2 weeks and bone replaced the cartilage in 3–4 weeks.¹¹

B. BONE DEFECT MODELS AND ANIMAL SELECTION

Selected bone defect models are listed in Table 2. There are mainly four types of defects including calvarial, long bone (or mandible) segmental, partial cortical (cortical window, wedge defect, or transcortical drill hole), and cancellous bone defect (drill holes). The calvarial defect and long bone segmental defect are used the most often. The commonly used animals are rabbits, rats, dogs and sheep. Rabbits and rats are first choice for calvarial models and rabbits and dogs for segmental defects. Dogs and sheep are often used for experimental conditions involving heavy internal or external fixations.

III. AUTHORS' PREFERRED MODELS

A. HETEROTOPIC MODEL (RAT SUBCUTANEOUS MODEL)

Potential osteogenic materials can be implanted subcutaneously or intramuscularly in the rat. If the material is paste-like it can be injected using a syringe. The common place for implantation is the SC tissues on the back or upper abdominal area. Figure 1 shows radiographically and histologically a new bone nodule formed five weeks after the injection of a collagen gel impregnated with several osteogenic GFs. Osteogenic materials can also be implanted in muscle, peritoneal cavity, or mesentery.

B. CALVARIAL DEFECT MODELS

1. Rabbit Calvarial Defect Model

The rabbit calvarial defect model is very popular and appropriate for the following reasons: (1) the calvarial bone is a plate which allows creation of a uniform circular defect that enables convenient radiographic and histological analysis; (2) the calvarial bone has a good size for easier surgical procedures and specimen handling; (3) no fixation is required because of the supports by the dura and the overlying skin; (4) the model has been well studied and is reproducible, which permits precise comparison of a variety of graft substances; and (5) it is relatively economical compared to dogs.

When a calvarial model is selected, the critical size of the defect (CSD) for the animal species has to be considered. CSD is defined as the smallest size of a calvarial defect which does not heal spontaneously when left untreated for a certain period of time (often six months) (Table 3).¹²⁰ The CSD of adult NZW rabbit calvarial defect is 15 mm.⁵⁴

For operative procedures, an anteroposterior midline skin incision (4–5 cm long) is made over the cranial vault. The periosteum is elevated and retracted to expose the cranial bone. A 15-mm diameter defect is created using a trephine (Figure 2A). The drill should be centered at a point on the midline 5 mm posterior to the transverse bone sutures between the frontal and parietal bones (Figure 2B). Copious saline irrigation is needed during the drilling. Care must be taken to avoid perforating the dura, which is usually achieved by time to time checking of the osteotomy depth. Usually, the bone disc needs to be taken out of the defect by breaking the left-over connection points in between the bone disc and the edge of the defect. A neural elevator is very helpful for checking the depth, breaking bone connections within the osteotomy, and separating the dura from the inner surface of the bone disc. The key is never cut the bone plate all way through. If this happens, it is very likely the dura has already been cut, which could cause an intracranial hematoma. After specific treatment, as often an implantation of a bone substitute (Figure 3), the periosteum is closed and strengthened by closing the adjacent muscles and SC tissue. Occasionally, a SC hemotoma may occur after surgery and it can be drained by cutting several skin stitches. Wound infection is rare.

TABLE 2
Animal Bone Defect Models Selected from the Literature

Animal	Bones	Types of defect	First author, year ^{Ref}
Rat	Cranial bone	Circular	Ray 1957, ²² Takagi 1982, ²³ Noda 1989, ²⁴ Hollinger 1991, ²⁵ Kenly 1994, ²⁶ Miki 1994, ²⁷ Gombotz 1994, ²⁸ Kobayashi 1995, ²⁹ Sweeney 1995, ³⁰ McKinney 1996, ³¹ el Montaser 1997 ³²
	Radius	Segmental	Herold 1971, ³³ Gepstein 1987, ³⁴ Alper 1989, ³⁵ Solheim 1992, ³⁶ Nyman 1995 ³⁷
	Femora	Segmental	Melcher 1962, ³⁸ Einhorn 1984, ³⁹ Pelker 1989, ⁴⁰ Wolff 1994, ⁴¹ Feighan 1995, ⁴² Langenskiold 1996, ⁴³ Hunt 1996, ⁴⁴ Puelacher 1996, ⁴⁵ Stevenson 1997 ⁴⁶
	Tibia	Metaphyseal window	Taguchi 1990, ⁴⁷ Uchida 1985 ⁴⁸
	Fibula	Segmental	Narang 1971, ⁴⁹ Chakkalakal 1994, ⁵⁰ Bluhm 1995 ⁵¹
	Nasal bone	Circular	Dupoirieux 1994 ⁵²
	Mandible	Circular	Zellin 1997 ⁵³
	Cranial bone	Circular	Frame 1980, ⁵⁴ Schmitz 1988, ⁵⁵ Damien 1990, ⁵⁶ Kleinschmidt 1993, ⁵⁷ Richardson 1993, ⁵⁸ Arnaud 1994, ⁵⁹ Meikle 1994, ⁶⁰ Robinson 1995, ⁶¹ Ashby 1996, ⁶² Rabie 1996 ⁶³
Rabbit	Radius or ulna	Segmental	Herold 1971, ³³ Tali 1981, ⁶⁴ Gupta 1982, ⁶⁵ Wittbjer 1983, ⁶⁶ Aspenberg 1986, ⁶⁷ Bolander 1986, ⁶⁸ Hopp 1989, ⁶⁹ Iyoda 1991, ⁷⁰ Yang 1994, ⁷¹ Ho 1995 ⁷²
	Femoral greater trochanter	Drill hole	Heikkilä 1995 ⁷³
	Femoral condyle	6 mm drill hole	Kühne 1994, ⁷⁴ Roudier 1995 ⁷⁵
	Upper femoral cortex	3.5 mm drill hole	McCormack 1993 ⁷⁶
	Tibia	Circumscribed	Shimazaki 1985, ⁷⁷ Suh 1987, ⁷⁸ Uchida 1985, ⁴⁸ Shimizu 1988 ⁷⁹
	Fibulae	Segmental	Yang 1994, ⁷¹ Taguchi 1995 ⁴⁷
	Scapular bone	Circular	Oikarinen 1978 ⁸⁰
	Mandible	Rectangular defect	Eppley 1988 ⁸¹
	Cranial bone	18–20 mm circular	Oklund 1986 ⁸²
	Radius or ulna	Segmental	Holmes 1987, ⁸³ Key 1934, ⁸⁴ Nilsson 1986, ⁸⁵ Moore 1987, ⁸⁶ Delloye 1990, ⁸⁷ Grundel 1991, ⁸⁸ Cook 1994, ⁸⁹ Johnson 1996, ⁹⁰ Sciadini 1997 ⁹¹
Dog	Radius	Cortical window	Bay 1993 ⁹²
	Ulna	Segmental/bioreactor	Frayssinet 1991 ⁹³
	Femur	Segmental	de Pablos 1994 ⁹⁴
		Wedge defect	Black 1990, ⁹⁵ St. John 1993 ⁹⁶
	Tibia	Segmental	Tiedeman 1989, ⁹⁷ Markel 1991 ⁹⁸
	Fibula	Segmental	Enneking 1975, ⁹⁹ Welter 1990 ¹⁰⁰
	Mandible	Segmental	Holmes 1979, ¹⁰¹ Toriumi 1991 ¹⁰²
	Cranial bone	18–20 mm circular	Lindholm 1988, ¹⁰³ Viljanen 1996 ¹⁰⁴
	Femur	Segmental	Ehrnberg 1993, ¹⁰⁵ Brunner 1994 ¹⁰⁶
	Tibia	6 mm drill hole	Hallfeldt 1995 ¹⁰⁷
Sheep		Segmental	Gao 1995 ¹⁰⁸
	Mandible	Holes	Gatti 1991 ¹⁰⁹
		Segmental	Stoll 1992 ¹¹⁰
	Femur	Transcortical hole	Radder 1996 ¹¹¹
Goat	Cranial bone	15 mm circular	Hollinger 1989 ¹¹²
Primate	Mandible	Grooves	Drury 1991 ¹¹³

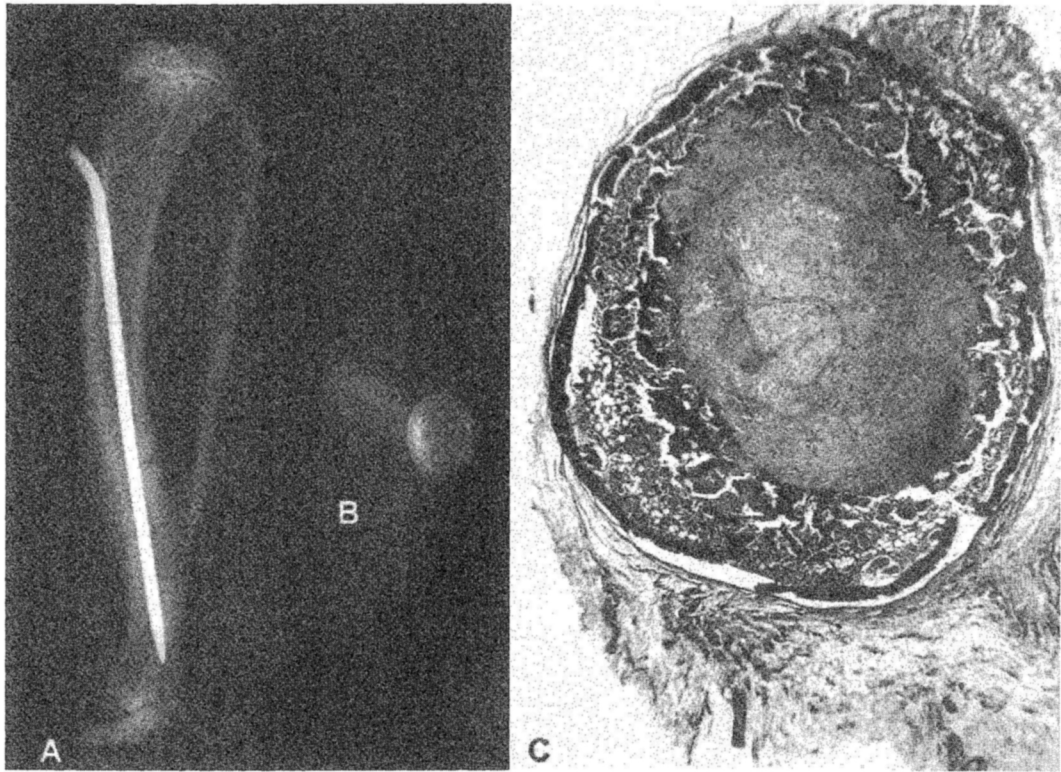


FIGURE 1. The radiographic (A,B) and histological (C) images of a new bone nodule five weeks after the injection of 100 μ l collagen gel impregnated with osteogenic GFs (undecalcified paraffin section with H&E staining). The histological section shows that a bony shell, containing a central area of fibrous tissue, has two layers with vascularized adipose tissue in between resembling marrow. In areas this osseous tissue is lined with osteoblasts overlying a layer of osteoid. Also there is a transition zone of cartilage between the bone and fibrous tissue.

TABLE 3
The Critical Sizes of Common Animal Calvarial Defect Models

Animal	Strains	Defect size (Diameter)	Observation period (No healing, month)	First author year ^{Ref.}
Rat	Charles River	4 mm	6	Mulliken 1980 ¹¹⁴
	SD	8 mm (CSD)	3	Takagi 1982 ²³
	—	8 mm	6 weeks	Schimitz 1990 ¹¹⁵
Rabbit	New Zealand White	8 mm	Healed in 4 weeks	Kramer 1968 ¹¹⁶
	NZreds-Half Lop*	15 mm (CSD)	6 and 9	Frame 1980 ⁵⁴
Dog	Mongrel	17 mm (CSD)	5	Friedenberg 1962 ¹¹⁷
	Mongrel	20 mm	6	Prolo 1982 ¹¹⁸
Sheep	In Finland, Europe	18–20 mm (CSD)	3	Lindholm 1988 ¹⁰³
Primate	Baboon	15 mm	2	Hollinger 1989 ¹¹²
		25 mm	3, 6, and 9	Ripamonti 1991 ¹¹⁹

* NZreds-Half Lop means the rabbits are crossbreeds of New Zealand reds and half lops.

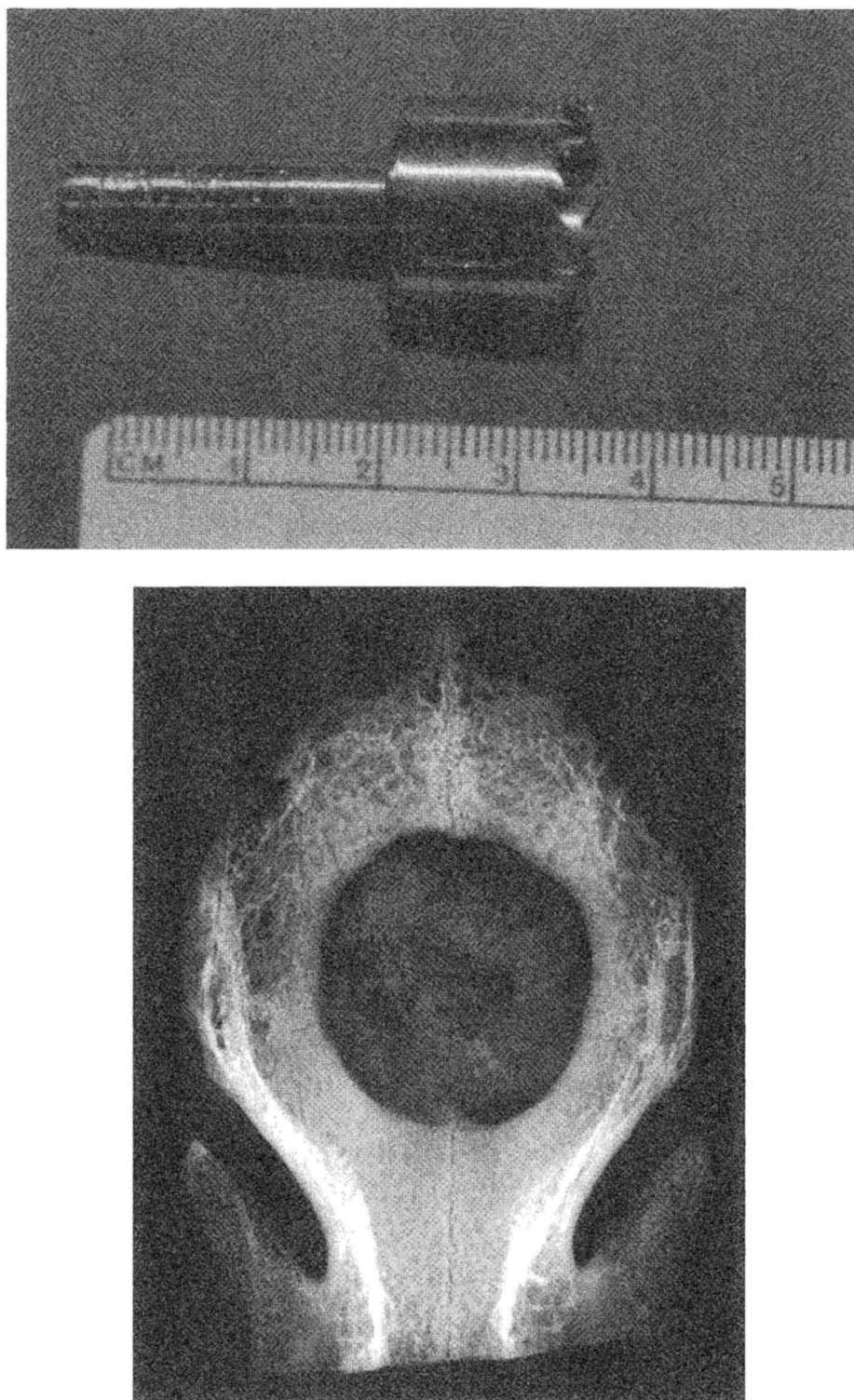


FIGURE 2. (A) Photograph of a trephine used for creating rabbit calvarial defect. (B) A defect three months after surgery showing no healing. It also shows where the trephine should be centered, which is the point on the midline 5 mm posterior to the transverse bone sutures between the frontal and parietal bones.

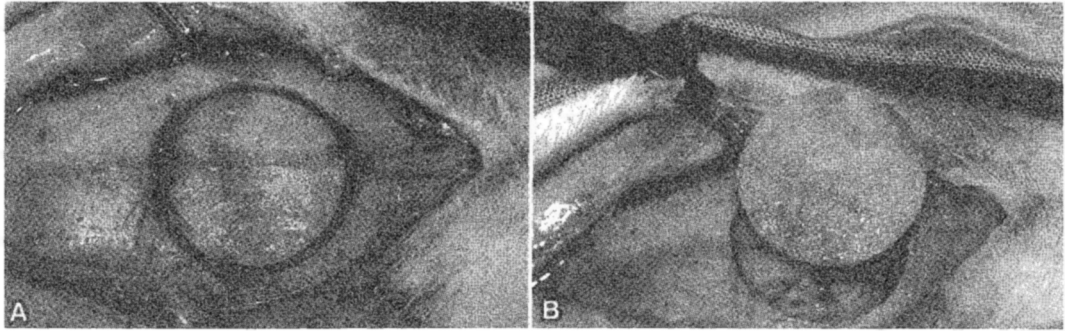


FIGURE 3. (A) A calvarial defect has been created with the bone disc still in place. (B) A disk implant (an HA-collagen complex) is ready to be placed in the defect.

2. Rat Calvarial Defect Model

The rat calvarial model is another popular one for the following reasons: (1) the calvarial bone is a plate which is large enough to allow creation of a circular defect that enables convenient radiographic and histological analysis; (2) the calvarial bone has a large enough size for easier surgical procedures and specimen handling; (3) no fixation is required; (4) the model has been well studied and is reproducible, which permits precise comparison of a variety of graft substances; and (5) it is much cheaper than rabbits. The CSD of a SD rat calvarial defect is considered as 8 mm in diameter (Table 3). There is one major concern about this model, which is the fast healing ability of the rat. The surgical procedure for the rat calvarial defect is about the same as that for rabbit. For the small size, a pair of operating glasses are essential.

C. LONG BONE DEFECT MODELS

1. Segmental Defects of Rabbit Forearm Bones

The rabbit radial model is popular and appropriate for the following reasons: (1) the radius bone is tubular, which allows creation of a segmental defect that enables convenient radiographic and histological evaluation; (2) the radius has a good size for easier surgical procedures and specimen handling; (3) no fixation is required because of the support of ulna; (4) the model has been well studied and is reproducible, which permits precise comparison of a variety of graft substances; and (5) it is relatively economical. The rabbit radial defect model was first described by Herold in 1971 to test the effect of growth hormone on the healing of bone defects.³³ It is well accepted that the CSD of long bones is two times the bone diameter. Because the diameter of the radius of adult NZW rabbits is about 5–6 mm, a radial defect should be no less than 12 mm long. In the author's laboratory, three out of twenty 12 mm defects healed spontaneously in 12 weeks. Therefore, it is the author's opinion that a 15 mm defect should be created.

Surgically, a longitudinal skin incision is made over the radial bone at the middle one third of the front leg. The periosteum is carefully separated from the surrounding muscles. A 15 mm defect located about 2.0 to 2.5 cm proximal to the radiocarpal joint (for the first cut) (Figure 4A) is created using a circular saw attached to a mini driver or dental handpiece (see Figure 3 in Chapter 11). The defect should be checked carefully and any periosteum left removed. A thorough wash with saline is needed before any treatment applied. The defect is then grafted with bone substitute or a spacer or left empty. No fixation is needed. Postoperatively, unrestricted weight bearing is normally allowed. Radiographs should be taken immediately after the surgery and also at any designated time periods. Figure 4B is a radiographic image showing a healed 15 mm radial defect by implantation of a porous form DBM in eight weeks. Adult rabbits with closed growth plate are preferred, which eliminated the possibility of epiphyseal slipping (Figure 5).

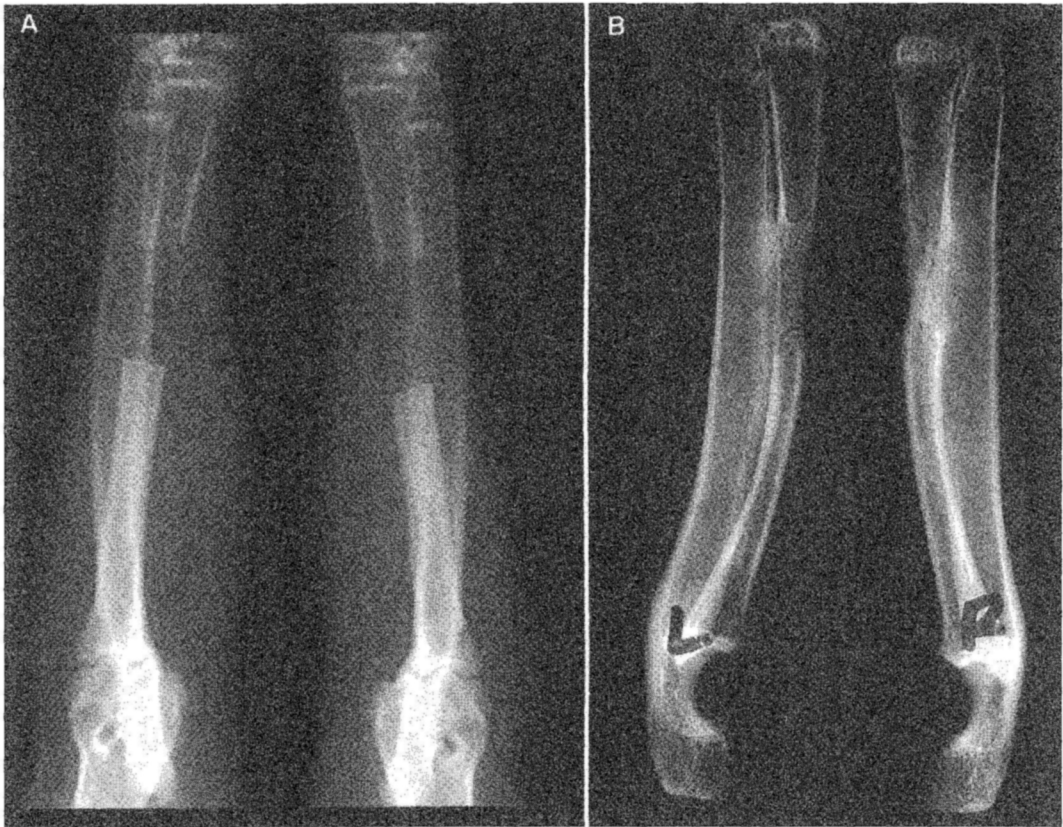


FIGURE 4. An X ray image showing a healed 15 mm radial defect by implantation of a porous DBM in eight weeks.

A rabbit ulnar defect is also popular, but the ulnae do not have the round shape as the radius, which creates difficulties and errors to the processes of implant preparation, implant positioning, and sample evaluation. Also because of its shape, doing mechanical testing, such as bending or torsional tests, on the ulnar bone is less favorable.

C. THE SECOND CHOICE

If there are reasons for not using the three models mentioned above, the rat femoral defect model and the dog radial model could be selected. The former is a good model with only one shortcoming, the need for a secured internal fixation or external fixator. Due to the small size of the rat limb, it can be very frustrating to try to put an effective fixation device on. The dog radial defect model is popular and appropriate. Occasionally, because of the active nature of the dog, a fracture of the ulnae may occur, resulting in a tremendous loss compared to a rat or rabbit model.

IV. EVALUATION OF BONE DEFECT REPAIR

A. RADIOGRAPHY

Using radiographic analysis, information on the amount and quality of the new bone, such as bone density and structure, and continuity with the adjacent recipient bone, can be obtained. In recent years, computerized image analysis makes this more efficient. Radiographs taken at regular intervals



FIGURE 5. The radiograph shows a distal ulnar epiphyseal slipping when a young rabbit was used.

after surgery provide continuous information on the process of bone healing. For a long bone segmental defect, healing parameters, such as periosteal reaction (callus formation), appearance of the graft (bone formation), quality of union at both osteotomies, and bone remodeling are quantitated based on a radiographic scoring system modified from the ones by Bos et al.,¹²⁰ Lane and Sandhu,¹²¹ Yang et al.,⁷¹ and Johnson et al.¹²²(Table 4).

TABLE 4
Radiographic Scoring System

Categories	Scores
Periosteal reaction*	
Full (across the defect)	3
Moderate	2
Mild	1
None	0
Proximal osteotomy union	
Union	3
Moderate bridge (>50%)	2
Mild bridge (<50%)	1
Nonunion	0
Distal osteotomy union	
Union	3
Moderate bridge (>50%)	2
Mild bridge (<50%)	1
Nonunion	0
Appearance of graft	
Full replacement	3
Moderate replacement (>50%)	2
Mild replacement (<50%)	1
No change	0
Remodeling	
Full remodeling of cortex	2
Intramedullary canal	1
No remodeling	0
Maximum total score	14

* For some osteogenic materials such as collagen sponge/GFs, this category may not apply because of the nearly absent periosteal reaction.

B. HISTOLOGY AND HISTOMORPHOMETRY

Histology is the most powerful method of examining the healing of bone defects. Common histological parameters include the following categories: bone union at the two osteotomies, callus formation, new bone formation in the defect, resorption of the bone graft, marrow changes, and cortex remodeling, which are semi-quantitated based on a scoring system modified from the ones by Bos et al.,¹²⁰ Nilsson et al.,⁸⁵ Lane and Sandhu,¹²¹ Heiple et al.,¹²³ and Suh et al.⁷⁸ (Table 5). Based on the nature of the graft to be used, the scoring system may be customized to suit the individual situation.

In recent years, computerized image analysis makes histomorphometry more efficient, especially for the calculation of the percentage of filling by the repair tissues in the defect and the fractions of different tissues in it. For histomorphometrical analysis of ectopic bone formation, the following elements can be quantified: area and penetration depth of mineralized bonelike tissue, area and thickness of nonmineralized bonelike tissue, area of osteoblast-covered surfaces, thickness of trabeculae, area and thickness of cartilage tissue, area of fibrovascular tissue, and void space.^{9,14} For analysis of repair tissues of bone defect the areas of mineralized bone, nonmineralized bone, lamellar bone, woven bone, chondral tissue, fibrocartilage, or fibrous vascular tissue can be used as parameters for quantification.^{41,124} See Chapter 6 for more information on bone histomorphometry.

TABLE 5
Histological Scoring System

Categories	Scores
Callus formation*	
Full (across the defect)	3
Moderate	2
Mild	1
None	0
Proximal osteotomy union	
Full bone bridge (union)	3
Moderate bridge (>50%)	2
Mild bridge (<50%)	1
No new bone in the osteotomy line (nonunion)	0
Distal osteotomy union	
Full bone bridge (Union)	3
Moderate bridge (>50%)	2
Mild bridge (<50%)	1
No new bone in the osteotomy line (nonunion)	0
Resorption of the bone graft	
Fully absorbed	3
Moderate adsorption (>50%)	2
Mild adsorption (<50%)	1
No change	0
New bone formation in the defect	
Full bone formation in the defect	3
Moderate bone formation (>50%)	2
Mild bone formation (<50%)	1
No new bone	0
Marrow changes	
Adult type fatty marrow	4
2/3 replaced by new tissue	3
1/3 replaced by new tissue	2
Fibrous tissue	1
Red	0
Cortex remodeling	
Full remodeling cortex	2
Intramedullary canal	1
No remodeling	0
Maximum total score	21

* For some osteogenic materials such as collagen sponge/GFs, this category may not apply because of the nearly absent periosteal reaction.

C. MECHANICAL TESTING

Mechanical tests, such as torsional test,^{40,69,71,87,89,95,122,125} bending test,¹⁰² and tensile strength¹²⁶ of the repaired site, have been used effectively to evaluate the mechanical properties of the repair. For long bone segmental defect models, torsional testing is an appropriate and the most popular method.

V. BONE SUBSTITUTES AND FUTURE DIRECTIONS OF RESEARCH

A. MECHANISMS OF BONE REPAIR BY BONE GRAFTING

The mechanisms of bone repair by bone grafting vary with the different types or composition of substitutes. Three basic mechanisms have been described for bone defect repair by grafting, osteoconduction, osteoinduction and direct osteogenesis. With osteoconduction, the grafted dead bone or bone substitute acts as a scaffolding for the ingrowth of blood vessels and new bone while itself being resorbed gradually by the host tissues. Osteoconduction is very important because without a scaffold defect, repair is less likely. Osteoinduction occurs when local mesenchymal cells, undifferentiated cells, or even muscle cells are transformed into bone-forming cells in the presence of certain stimulators, such as growth factors or hormones.¹²⁷ Direct osteogenesis is regulated by autogenic osteoblasts implanted with the graft or from the edges of the defect. Bone graft substitutes which can provide all three mechanisms for osteogenesis would be considered ideal bone grafts, since these mechanisms are often absent or insufficient at the sites of bone defect of nonunion and delayed union.

B. AUTOGRAFT, ALLOGRAFT, AND XENOGRAFT

In 1923, Albee reported 3000 cases of successful bone grafting.¹²⁸ In 1944, the use of iliac crest bone grafts in 75 cases were reported.¹²⁹ Since then, fresh autogenous bone grafts have been the most successful for skeletal reconstruction. The reasons for the high success rate include the osteoconductive ability for the ingrowth of blood vessels and new bone, the presence of pre-existing differentiated osteogenic cells for immediate osteogenesis, the existing growth factors for osteoinduction, and the lack of immunologic rejection from the host. Unfortunately, autogenous bone graft cannot solve all osseous defects because of its limited quantity, donor site morbidity, and in some situations, the difficulty of fabricating the graft into a desired shape.

Despite the reported potential rejection from the host,¹³⁰ allogenic grafts are well tolerated, nontoxic, incorporated by host bone, and last a long period of time.¹³¹ Due to its availability, allograft bone is one of the major methods for bone grafting in certain conditions, such as revision total joint arthroplasty, tumor surgery, or limb salvage.

Xenograft alone tends to fail (due to inflammatory reaction) in a rat model compared to allograft.¹³² In another animal study, decalcified xenogenic bone impregnated with fresh autologous marrow was reported bridging a large cortical defect successfully.¹³³ Clinically, xenograft of hydrogen peroxide-macerated bone (Kiel bone) in combination with autologous marrow healed most of the bone defects or pseudoarthroses in two series.^{134,135}

C. DEMINERALIZED BONE MATRIX

By chemical or physical methods, allogenic, xenogenic, or even autogenous bone can be demineralized to make DBM which can be fabricated into powder or porous form or molded into certain solid shapes. DBM has been studied extensively since the early 1960s.^{22,35,39,64,80,136} The osteogenic mechanisms of DBM include osteoconduction^{137,138} and osteoinduction.^{13, 16} Because of its availability, diverse uses, and limited immunological response, DBM has a bright future in research and clinical practice for repairing bone and perhaps cartilage defect.¹³⁹

D. BIOMATERIALS

Biomaterials such as ceramics, polymers, or metals have been investigated extensively as bone substitutes with varying degrees of success. Hydroxyapatite,^{35,92} tricalcium phosphate (TCP),⁷⁷ and composite forms such as HA/TCP ceramic⁸⁶ have been used for bone defects. The rate of bone regeneration into material pores varies depending upon the implant location, the availability of

local osteogenic cells, and the stability of the implant. Clinical results reported by Bucholz et al.¹⁴⁰ showed that filling traumatic and tumor defects with HA and TCP healed in most of the cases. These materials act as osteoconductive scaffolds and have no osteoinductive ability. Attention has been paid to adding growth promoting factors such as GFs,¹⁴¹ bone marrow,^{88,142} or DBM³⁶ to the implants.

E. BONE MARROW

Adding autogenous bone marrow to bone substitutes (allograft, xenograft, DBM, or biomaterials) has been investigated since the early 1960s.^{88,142–144} The purpose is to increase the osteogenic capability of the graft with osteogenic precursor cells which differentiate into bone forming cells in the recipient site.¹⁵ Autogenous marrow is now being used clinically as an osteogenic material for repairing skeletal defects.^{137,143}

F. GROWTH FACTORS

Bone tissue harbors many growth factors (GFs), such as bone morphogenic protein (BMP), transforming growth factor (TGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF). It is known that bone structure is maintained through a balancing process of bone formation and resorption, and is mediated by cellular activities and regulated by systemic hormones and local GFs. GFs have the functions of stimulating osseous cellular proliferation, differentiation, DNA and protein synthesis, and extracellular matrix synthesis. They either act alone or in combinations. In the last two decades, a large number of *in vitro* and *in vivo* studies have been done and there has been significant evidence supporting the use of GFs for bone repair.^{145,146}

G. TISSUE ENGINEERED COMPOSITE GRAFT

Bone grafting is aimed to provide the missing elements necessary for bone formation in a bone defect and thereby restore the bone integrity. Using cell-seeding to a substrate to make an implantable graft is not a new concept.^{147,148} Recently, a few groups reported some preliminary and very important data on the use of cell-seeded implants for repairing osseous or chondral defects.^{93,149–151} In 1991, Frayssinet et al. reported that bone cells from canine humeri were grown on HA granules and the cell-HA composite was placed in a bioreactor and implanted into a canine ulna defect. Osteogenesis was seen in active bioreactors three weeks after implantation.⁹³ Cultured chondrocytes bound to a HA block were implanted to repair a rabbit ulna defect.⁷⁰ Osteoblast-like cells (MC3T3-E1) were also used to study the potential of bioabsorbable polymers and ceramics to support osteoblastic growth for a bone-polymer composite in bone repair.¹⁵⁰

The above-mentioned studies demonstrate that cell-seeded composite implants can induce bone tissue formation, leading to defect repair. Osteogenic cell-seeded composite implants act in a similar way as bone marrow transplantation does, and this technique has been referred to as tissue engineering.¹⁵² Over other methods, it has certain advantages, such as the autogenic characteristics (if cells come from the same individual), minor morbidity of the donor site, no risk of disease transmission, and permitting potential production of a large quantity *in vitro*.

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14 Animal Models of Osteonecrosis

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I. INTRODUCTION

It has long been suggested that nontraumatic osteonecrosis (ON) of the femoral head may be associated with corticosteroid therapy, alcohol abuse, smoking, systemic lupus erythematosus, and renal transplantation. While much has been learned concerning the complex characteristics of nontraumatic ON, no means are yet available to prevent this disorder, and attempts to clarify pathogenesis of ON have been hampered by a number of problems. Incomplete early diagnosis of this disease, and the lack of useful experimental animal models of ON have prevented complete characterization of the pathological conditions present prior to the development of ON. With the advent of high performance magnetic resonance imaging (MRI) and optimized coil technology, an opportunity has arisen to detect subtle structural abnormalities in bone, enabling early diagnosis of bone diseases. Accordingly, more reproducible animal models of ON are needed in order to clarify the etiology and early pathogenesis of ON.

In the 1960s, 1970s, and 1980s, many attempts were made to induce ON traumatically in experimental animals such as intra-arterial infusion of oil (Lipiodol),¹ ligation of the blood vessels feeding the femoral epiphysis,^{2,3} and intracapsular tamponade in the hip joint using wax or silicone.^{4,5} However, the canine and rabbit models of ON produced by these methods cannot serve as animal models of nontraumatic ON in humans. In the 1990s, several animal models of nontraumatic



FIGURE 1. Femoral head necrosis in SHR ($\times 40$).

ON have been successfully produced, from which a number of essential pathological findings have been obtained. The historical background, experimental protocol and principal findings obtained from studies using different animal models will be described below.

II. NONTRAUMATIC MODELS OF OSTEONECROSIS

A. SPONTANEOUSLY HYPERTENSIVE RAT (SHR) MODEL

1. Introduction

Skeletal disorders in SHR including osteoporosis and abnormal calcium metabolism have been reported, in addition to disorders of the endocrine and autonomic nervous systems.^{6,7} In the late 1980s, it was incidentally observed that widespread ON frequently developed naturally in the epiphysis of the femoral head in growing SHR. In SHR with systemic skeletal growth retardation, ON naturally occurs in growing male rats and heals without remarkable deformity. These findings obtained in preliminary studies by Hirano et al. suggested that ON in SHR closely resembles Perthes' disease.^{8,9} The site and mechanism of the occlusion of the blood vessels feeding the femoral head were examined histologically and microangiographically by Iwasaki et al.¹⁰

2. Materials and Methods

One hundred fifteen growing male SHR (230 femurs) were used.¹⁰ The femurs were fixed in formalin and decalcified with formic acid and hydrochloric acid before being embedded in paraffin. Thin coronal sections of the proximal femur were prepared and H&E stained. Histologic examination revealed three different sets of characteristic findings for the femoral head: (1) normal ossification (75/230); (2) abnormal (disturbed) ossification (78/230); and (3) ON (77/230). The group with ON was divided into subgroups based on stage of the healing process:

- Stage 1** Fresh necrosis of trabeculae and bone marrow without repair tissue (Figures 1, 2)
- Stage 2** Invasion of vascular granulation tissue into the intertrabecular space
- Stage 3** Completely repaired ON with empty lacunae in the center to hypertrophic trabeculae

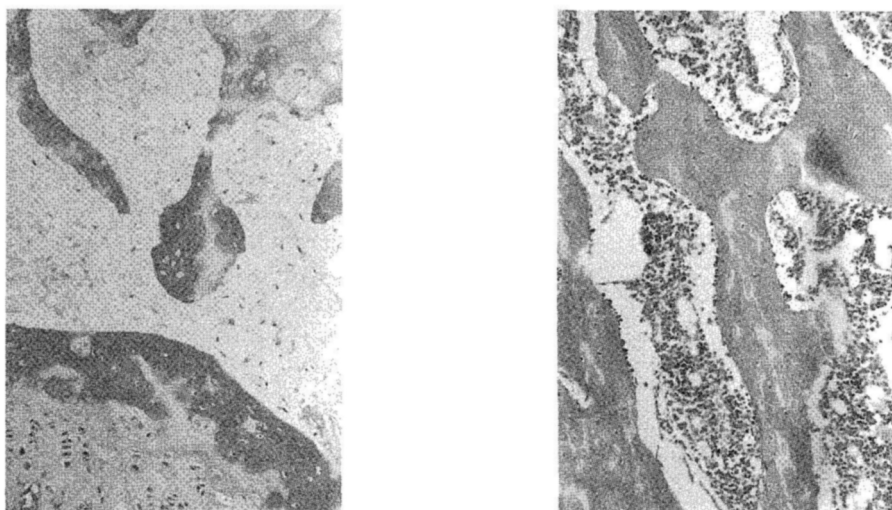


FIGURE 2. (A): Fresh necrosis of trabeculae and bone marrow without repair tissue in the epiphysis ($\times 200$). (B): Normal structure of trabeculae and bone marrow in the metaphysis ($\times 200$).

Microangiography using Micropaques solution¹¹ was performed on 30 male SHR rats (10- to 40-week-old rats).

For assessment of the relationship between femoral head lesions and mechanical stress on the femoral head, 38 male SHR and 10 male WKY rats, each 6-weeks-old, were divided into five groups: A: no treatment, 10 WKYs (genetic control); B: no treatment, 10 SHRs; C: bilateral severance of the sciatic nerves, 8 SHRs; D: bilateral severance of the sciatic and femoral nerves, 10 SHRs; and E: amputation of the right lower hind limb, 10 SHRs.

After each treatment, the rats were killed at the age of 15 weeks and examined to determine the incidence of femoral head lesions such as ON, disturbed ossification, and abnormalities of the growth plate.

3. Essential Findings

In the femoral heads with fresh ON (Stage 1), the lateral epiphyseal vessels disappeared before entering the epiphysis. In the femoral heads with invasion of vascular granulation tissue (Stage 2), many slender vessels and capillaries entered the ossifying nucleus with the granulation tissue. In the femoral heads with completely repaired ON (Stage 3), blood vessels corresponding to the lateral epiphyseal vessels in normal femoral head entered the ossified nucleus.

Homogeneous distribution of blood vessels in the epiphysis was observed in the femoral heads with normal ossification and in heads with completely repaired ON (Stage 3), while absence of blood vessels throughout the epiphysis was demonstrated in the femoral heads with fresh ON without repaired tissue (Stage 1) and in the femoral heads in which no ossification had occurred at all. These findings suggest that ON and disturbed ossification may be the result of the same pathogenetic process.

The rats of Group C were able to walk using their hind limbs, in which quadriceps muscle function was intact. In the rats of Group D, active movement of the hind limbs was not observed. Locomotion was achieved using only the forelimbs, with dragging of the hind limbs. In the rats of Group E, the hip on the treated side was kept in flexion, and the amputated stump was suspended in midair. These observations suggested that the degree of stress on the femoral head was the highest in Groups A and B and the lowest in Groups D and E, Group C being intermediate. The incidence of ON, disturbed ossification, and growth plate abnormalities of the femoral head in each

TABLE 1
Incidence of ON, Disturbed Ossification and Growth Plate Abnormality

	Group A (n = 20)	Group B (n = 20)	Group C (n = 16)	Group D (n = 20)	Group E	
					Amputation (n = 10)	Nonamputation (n = 10)
Osteonecrosis	15%	60%	25%	0%	0%	40%
Disturbed ossification	0%	25%	12.5%	0%	0%	10%
Growth plate abnormality	15%	85–100%	50–69%	30%	30%	80–90%

(Adapted from Reference 10)

group are summarized in Table 1. Decrease in the incidence of femoral head lesions, such as ON, disturbed ossification, and abnormality of the growth plate accompanied a decrease in mechanical stress on the femoral head.

B. STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RAT (SHRSP) MODEL

1. Introduction

Marked hypertension and cerebral apoplexia are more common in SHRSPs than in SHRs. Histopathologically, SHRSPs often exhibit an age-dependent increase in ischemic lesions such as infarction in the brain, kidney, and heart, and some antihypertensive drugs completely prevent the hypertensive complications induced by angiospasm or arteriosclerosis in SHRSPs. Naito et al. reported that SHRSPs had high incidence of femoral head necrosis.¹²

2. Materials and Methods

A total of 135 male rats six to 36 weeks of age, including 40WKYs (genetic control), 40 SHRs, and 55 SHRSPs, were used.¹² The length from the top of the major trochanter to the distal end of the femur was measured with a Vernier micrometer. The femur was placed in a small plastic phantom, and its bone mineral density (BMD) was measured using dual energy X ray absorptiometry (QDR-1000, Hologic). Portions of the femoral head, including the epiphyseal growth plates, of SHRSPs, SHRs, WKYs were resected at 12 weeks of age from the femur and subjected to a compressive load with the apparatus (Autography DCS-500, Shimazu, Japan). The hemispherical femoral head was placed between the plates, and a compressive load was administered vertically. The minimal compressive load at which the femoral head began to deform was recorded. The proximal femurs were fixed in 10% formalin solution and prepared for paraffin embedding after decalcification in EDTA. Thin sections through the teres ligament were stained with hematoxylin-eosin and toluidine-blue and examined by light microscopy.

3. Essential Findings

Among the 12-week-old rats, the mean bone length was least in SHRSPs and greatest in WKYs. This difference was statistically significant, but there were no significant differences in bone length among the groups after 12 weeks of age. The lowest mean femoral BMD was observed in SHRSPs and the highest in WKYs at all ages, while the BMD in SHRs was intermediate.

The mean compressive loads necessary to cause deformation of femoral heads in 12-wk-old SHRSPs, SHRs, and WKYs were 11.6 ± 1.2 kg, 13.2 ± 1.5 kg and 14.7 ± 0.9 kg, respectively. The femoral heads in SHRSPs were the most easily deformed by loads applied during compression tests.

TABLE 2
Incidence of Femoral Head Necrosis in
SHRSPs as Compared to SHRs and WKYs

Strain	10–14 weeks	20–36 weeks
SHRSP	70%	45%
SHR	53.3%	20%
WKY	20%	13.3%

(Adapted from Reference 12)

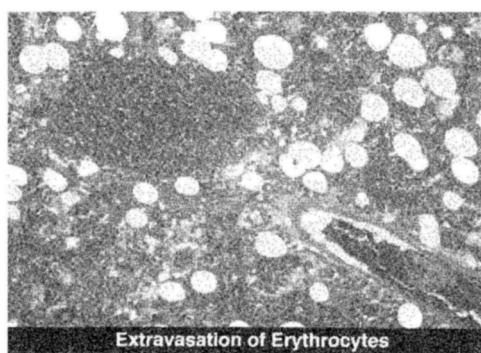


FIGURE 3. Extravasation of erythrocytes in bone marrow in the metaphysis in a rabbit with hypersensitivity. ($\times 200$)

Femoral head necrosis was observed in ossified regions with vascular invasion. In osteonecrotic regions in the young SHRSPs, dead trabeculae exhibited many empty lacunae without osteocytes and loss of marrow tissue. In osteonecrotic regions in the old SHRSPs, proliferation of the repairing tissue was observed. Empty lacunae were concentrated in the center of trabeculae, while the periphery of trabeculae was occupied by newborn osteocytes and osteoblasts. Infarctions were encountered on the lateral side of the epiphysis, but no thrombi were observed in any of the necrotic lesions. Femoral head necrosis was usually present in SHRSPs and SHRs from a young age (from about eight weeks). The incidence of femoral head necrosis in SHRSPs was 70% from 10 to 14 weeks of age and 45% from 24 to 36 weeks of age. The incidence of femoral head necrosis was highest in SHRSPs at all ages as shown in Table 2.

C. STEROID-TREATED RABBIT MODEL

1. Introduction

Studies have been conducted in corticosteroid-treated rabbits in more than 10 different laboratories, and have suggested possible pathogeneses of corticosteroid-induced ON such as increase in the size of fat cells,¹³ fatty degeneration of osteocytes,¹⁴ subchondral fat embolism in the femoral head¹⁵ and focal osteocytic death in subchondral bone.^{16,17,18} However, these studies never successfully produced histologically definitive ON in animals by injection of corticosteroid alone. In a more recent study, Yamamoto et al. reported that a single high-dose injection of corticosteroid induced thrombocytopenia, hypofibrinogenemia, and hyperlipemia with multifocal ON in several bones.¹⁹

2. Materials and Methods

Twenty-six male adult (Japanese white) rabbits weighing 3.0–4.5 kg were injected once with 20 mg/kg body weight of methylprednisolone (MPSL) acetate into the right gluteus medius muscle.¹⁹ Rabbits were sacrificed at four, six, eight, and 10 weeks after the injection of MPSL.

Bone samples were fixed with 10% phosphate buffered formalin, pH 7.4, for one week and decalcified in 25% formic acid for three days. The specimens were embedded in paraffin, sectioned and were routinely stained with H&E, elastica van Gieson and Masson trichrome.

Bone samples from the femur and humerus were histopathologically examined for the presence of hematopoietic cell necrosis (cytolysis, karyorrhexis, or karyolysis), fat cell necrosis (the loss of either nuclei or distinct cell borders) and ON. ON was blindly assessed by three pathologists, based on the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes in the bone trabeculae, accompanied by surrounding bone marrow cell necrosis.

Hematologic and chemical examinations were performed to determine plasma levels of blood platelet, fibrinogen, free fatty acid, triglyceride, cholesterol, glutamine-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) in all animals before and after injection of MPSL.

3. Essential Findings

ON was observed in none of the rabbits in the control group, while multifocal osteonecrotic lesions were recognized in both femur and humerus at four weeks after steroid administration. ON was mainly found in the metaphysis and diaphysis, and was not in the epiphysis. In the femurs, the prevalence of ON was 43% at four weeks, 13% at 6, 25% at 8, and 25% at 10 weeks. The incidence of ON in the femoral condyle was 50% at four weeks. ON gradually accompanied the repair process after six weeks, and the necrotic bone marrow was sometimes almost entirely replaced by repair tissue by 10 weeks after injection.

Histopathologically, typical ON featured accumulation of bone marrow cell debris and bone trabeculae demonstrating empty lacunae occasionally containing some pyknotic nuclei of osteocytes. Repair tissue such as granulation tissue and appositional bone around ON varied based on the number of weeks after corticosteroid injection. At four weeks, little granulation tissue was present surrounding ON. At six weeks, fibrosis and vascular- or cellular-rich granulation tissue surrounded the necrotic area, while whether appositional bone formation was present was still unclear. At 10 weeks, necrotic bone tissue was surrounded by prominent appositional bone formation and dense fibrotic granulation tissue.

Platelet counts and fibrinogen levels were decreased significantly one week after the injection of MPSL, and then gradually recovered and reached or slightly surpassed normal levels after five weeks. On the other hand, plasma FFA, triglyceride, cholesterol, GOT and GPT levels were significantly increased at two weeks and then gradually returned to normal by eight weeks. At 10 weeks, no abnormal findings were obtained for any of the items examined.

D. RABBITS WITH ENDOTOXIC (SHWARTZMAN) REACTIONS

1. Introduction

There have been reports that bacterial endotoxin reactions may cause osteonecrosis in humans by inducing disseminated intravascular coagulation.^{20–22} Yamamoto et al. have confirmed these clinical observations in rabbits in which corticosteroid was used to potentiate the Shwartzman reaction and increase the magnitude of ON.²³

2. Materials and Methods

Male adult NZW rabbits weighing 3.0 to 4.5 kg were used in the following groups: A: 100 µg/kg of endotoxin (lipopolysaccharide [LPS] from *Escherichia coli*) intravenously twice at an interval of 24 h, 10 rabbits; B: 20 mg/kg of MPSL intramuscularly three times at intervals of 24

TABLE 3
Prevalence and Location of Femoral Bone Necrosis

Group	n	Epiphysis	Metaphysis	Diaphysis
A	10	0%	20%	0%
B	14	29%	86%	79%
C	12	0%	33%	0%
D	10	0%	0%	0%

(Adapted from Reference 23)

hours after LPS administration as in Group A, 10 rabbits; C: 20 mg/kg of MPSL intramuscularly three times at intervals of 24 h, six rabbits; and D: no treatment, five rabbits.

Bone samples were fixed with 10% formalin-0.1 M phosphate buffer, pH 7.4, decalcified in 25% formic acid and then neutralized with sodium sulfate buffer. The specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin, elastica van Gieson, and Masson trichrome.

All bone samples were examined histopathologically for the presence of hematopoietic cell necrosis (cytolysis, karyorrhexis, or karyolysis), fat cell necrosis (the loss of either nuclei or distinct cell borders), and ON. ON was assessed based on histopathologic features of bone necrosis (the presence of empty lacunae or pyknotic nuclei of osteocytes and bone marrow cell necrosis). Evidence of repair, such as the presence of granulation tissue, fibrosis, or appositional bone formation, was also examined.

3. Essential Findings

Histologically, necrotic regions exhibited an accumulation of cell debris, disappearance of hematopoietic and fat cells, and bone trabeculae either demonstrating empty lacunae or containing pyknotic nuclei. The formation of granulation tissue representing repair around ON varied among groups and depended on whether or not the rabbits received steroid. In Group A without steroid treatment, granulation tissue was well formed around regions of ON. In Group B with steroid treatment, the extent of formation of granulation tissue was less than in Group A. In Group C, with steroid treatment only, no formation of granulation tissue was observed, whereas exudative reaction (accumulation of serofibrinous exudate) was present around necrotic regions.

The incidence of ON was significantly higher in Group B than in the other groups (Table 3). In Group B, ON was frequently seen in the metaphysis (85.7%) and diaphysis (78.6%), and, occasionally, in the epiphysis (28.6%). No animal exhibited ON only in the epiphysis or diaphysis. In Groups A and C, ON was observed only in the metaphysis (20% and 33.3%, respectively). The distribution of the necrotic area in Group B was significantly wider than that in any other group.

E. RABBITS WITH HYPERSENSITIVITY REACTIONS (STUDY 1 BY THE AUTHORS)

1. Introduction

We successfully developed a rabbit model of ON.^{25,26} This model is characterized by early microcirculatory injury (extravasation of erythrocytes and microthrombi in arterioles of the femoral metaphysis in the early stage) (Figures 4, 5) and immune complex deposition in the kidney. We determined serial changes in histological features of our rabbit model of inducible ON and examined whether immune complexes can be detected in femoral bone marrow, and whether immune complex deposition is present surrounding osteonecrotic regions and is related to early microcirculatory injury adjacent to ON.²⁷

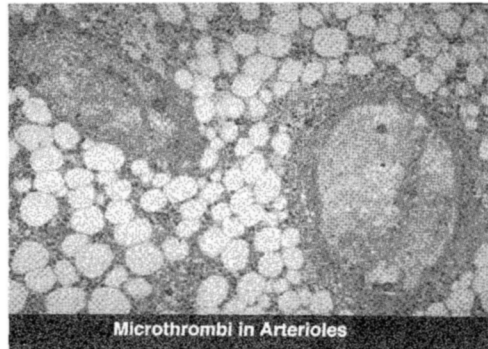


FIGURE 4. Microthrombi in arterioles in bone marrow of the femoral metaphysis in the same rabbit ($\times 200$).

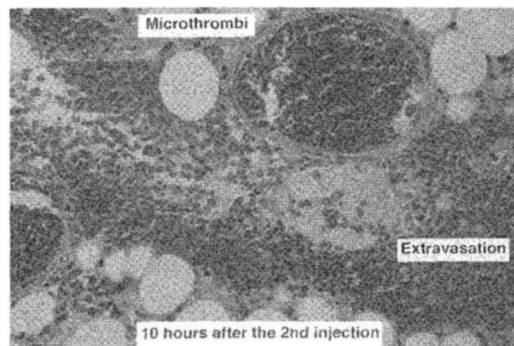


FIGURE 5. Major histological features including extravasation of erythrocytes and microthrombi in arterioles at 10 hours after the second injection of horse serum ($\times 200$).

2. Materials and Methods

One hundred and nine mature Japanese white rabbits, weighing 3.0–3.5 kg were used.²⁷ Whole horse serum was heat-inactivated at 56°C for 30 min. Ten ml/kg of heat-inactivated horse serum was given intravenously, and the same amount was injected again three weeks later.

Experimental Groups :

- Group A** Sacrificed prior to the second injection (at three weeks after the first injection), 17 rabbits.
- Group B** Sacrificed from one to four hours after the second injection of horse serum, 17 rabbits.
- Group C** Sacrificed from four to 12 hours after the second injection of horse serum, 17 rabbits.
- Group D** Sacrificed from 12 to 24 hours after the second injection of horse serum, 17 rabbits.
- Group E** Sacrificed from 24 to 72 hours after the second injection of horse serum, 21 rabbits.
- Group F** Sacrificed at one week after the second injection of horse serum, 20 rabbits.

The femurs and kidneys were excised bilaterally and fixed in buffered 4% paraformaldehyde saline (pH 7.4) at 4°C. The femurs were decalcified in EDTA (pH 7.4) at 37°C. These specimens were embedded in paraffin, sectioned, and stained with H&E stain. Phosphotungstic acid hematoxylin (PTAH) was used to demonstrate thrombi. In bone marrow, the presence of cytolysis, karyorrhexis, and karyolysis of marrow cells, and loss of nuclei and distinct cell borders of adipocytes was defined as bone marrow necrosis. We defined trabecular bone necrosis as being present when entirely empty lacunae of osteocytes were observed in the microscopic field at 100X magnification.

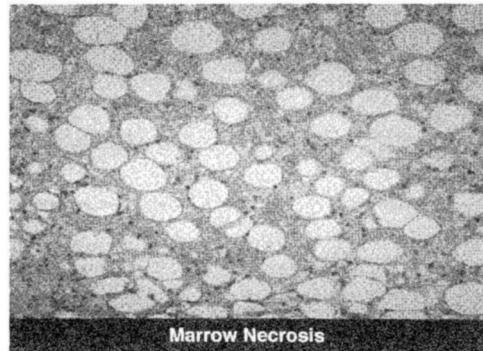


FIGURE 6. Bone marrow necrosis exhibiting necrotic debris and fibrosis at one week after the second injection of horse serum ($\times 100$).

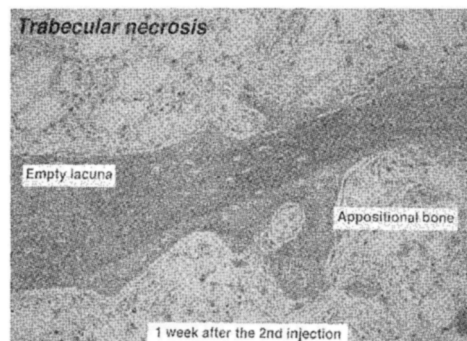


FIGURE 7. Trabecular bone necrosis surrounded by living appositional bone at one week after the second injection of horse serum ($\times 100$).

The femurs were embedded in paraffin, serially sectioned, and stained with the avidin-biotin-peroxidase complex method to detect immune complexes in bone marrow. An immunofluorescence method was performed to detect immune complexes in renal glomeruli.

3. Essential Findings

In Group A, neither extravasation of erythrocytes nor arteriolar microthrombi were observed in bone marrow of the femoral metaphysis. No necrosis of bone marrow and trabecular bone was observed immediately before the second injection of horse serum. Within 72 h after the second injection of horse serum (Group B, C, D, and E) major histological features included extravasation of erythrocytes (31%), arteriolar microthrombi (41%), and immune complex deposition in bone marrow of the femur. Extravasation of erythrocytes correlated well with the presence of arteriolar microthrombi ($p=0.0001$). In Group F, bone marrow necrosis and bone marrow replacement by fibrosis were observed in the femoral metaphysis in nine of 20 rabbits (45%) (Figure 6). Trabecular bone necrosis adjacent to bone marrow necrosis was observed in six of the nine rabbits (67%) with marrow necrosis, and necrotic trabeculae were surrounded by living appositional bone (Figure 7).

Immune complexes were demonstrated immunohistochemically in bone marrow (Figure 8) as well as in renal glomeruli (53%). Immune complex deposition both in the sinusoidal space of femoral bone marrow ($p=0.0385$) and in the renal glomeruli ($p=0.0209$) associated with extravasation of erythrocytes correlated well with the presence of arteriolar microthrombi in the early stage of this model. Early microcirculatory injury associated with immune complex deposition was present surrounding osteonecrotic regions.

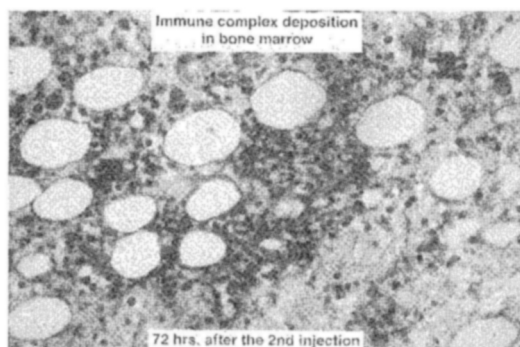


FIGURE 8. Immune complex deposition in the sinusoidal space of the femoral metaphysis ($\times 100$).

F. RABBITS WITH HYPERSENSITIVITY REACTIONS (STUDY 2 BY THE AUTHORS)

1. Introduction

We have developed a rabbit model of inducible ON.^{25,26,27} In this model, we reproducibly observed many characteristic histological features quite similar to those of clinical ON. Intravascular coagulation of the intraosseous microcirculation, potentially activated by various factors, has been suggested to play an important role in the genesis of nontraumatic ON.²⁰ We examined whether any abnormalities related to coagulation occur early in ON.

2. Materials and Methods

Eighteen Japanese white rabbits weighing 3.0–3.5 kg were used. Whole horse serum was heat-inactivated at 56°C for 30 min. Ten ml/kg of sterile heat-inactivated horse serum was administered intravenously using the method of Rich and Gregory.²⁸ The horse serum (10 ml/kg) was intravenously administered to rabbits, and the same amount was given again three weeks later. The animals were sacrificed one week after the second injection of horse serum.

The femurs were excised bilaterally, fixed in buffered 4% paraformaldehyde saline (pH 7.4) at 4°C and decalcified in EDTA (pH 7.4) at 37°C. The femurs were cut along the sagittal plane for observation of trabeculae and bone marrow. The specimens were embedded in paraffin, sectioned and stained with H&E. The incidence of ON in histological sections of the femur was determined. We classified cases into two groups (ON group or Non-ON group) based on whether or not osteonecrosis or marrow necrosis in femoral bone was present.

Blood samples were serially obtained immediately before and at 0.5, 1, 2, 6, 24, 48 and 72 hours after the second injection of horse serum. Total platelet, leukocyte and erythrocyte counts, thromboxane-B2 (TXB2), blood viscosity, and blood coagulation and fibrinolysis [prothrombin time (PT) and activated partial thromboplastin time (APTT)] were examined.

Rabbit platelet-rich plasma (PRP) was prepared by centrifugation (1500 rpm at room temperature, 10 min) of blood collected from the carotid artery of rabbits, with 1/10 v/v 90 mM trisodium citrate used as an anticoagulant. Aggregation was measured with a dual channel aggregometer linked to a dual pen recorder at 37°C and a stirring rate of 1100 rpm. PRP was pre-incubated with stirring at 37°C for 2 min. before the addition of stimulator. Platelet activating factor (PAF-C16: 10^{-7} M) (Bachem, California, USA) was used as aggregation stimulator. The rate of change in light transmission caused by platelet aggregation was recorded, and the maximal rate was considered a measure of platelet aggregation.

3. Essential Findings

Histologically, ON was demonstrated in six of 18 (33%) rabbits. Total platelet counts decreased within 0.5 hour and remained low for 48 hours after the second injection. There were significant

TABLE 4
Total Platelet Count

Total Platelet Counts ($\times 10^5/\mu\text{l}$)	Time after the second injection (hours)							
	pre	0.5	1	2	6	24	48	168
ON group (n = 6)	4.4	1.8	1.9	1.8	1.7	1.8*	2.1	3.6
Non-ON group (n = 12)	3.7	2.4	2.5	2.7	3.2	3.5*	3.1	3.3

* Significant (unpaired t-test)

TABLE 5
Level of TXB2

TXB2 ($\times 10^3/\mu\text{l}$)	Time after the second injection (hours)							
	pre	0.5	1	2	6	24	168	
ON group (n = 6)	0.8	6.7	11.4	19.1*	22.7*	2.3	1.0	
Non-ON group (n = 12)	0.6	6.6	6.5	2.5*	1.2*	1.3	0.5	

* Significant (unpaired t-test)

TABLE 6
Platelet-Aggregating Property

Platelet-aggregating property (%)	Time after the second injection (hours)							
	pre	0.5	1	2	6	24	168	
ON group (n = 6)	41	35	36	55*	42	39	36	
Non-ON group (n = 2)	45	37	37	36	38	41	33	

* Significant (unpaired t-test)

differences in total platelet counts at 24 and 48 hours between ON group and Non-ON group (Table 4). TXB2 was maximal at six hours after the second injection of horse serum in the ON group (2.3×10^4 pg/ml), and was significantly higher than in the Non-ON group (Table 5). Platelet aggregation was transiently increased at six hours after the second injection of horse serum in the ON group (55%) (Table 6). There were no significant differences between the two groups in leukocyte count, erythrocyte count, PT, or APTT.

III. TRAUMATIC MODELS OF OSTEONECROSIS

There is a need for more reliable treatment modalities for both traumatic and atraumatic ON. The clinical picture and histology of traumatic ON of the femoral head (ONF) resemble those of nontraumatic ONF. For transcervical fracture of the femur, the incidence of ONF and the area of infarction become larger as the severity of injury (Garden stage) increases. Traumatic ONF occurs in 11–16% of patients with stage I or II fracture and in 20–28% of patients with stage III or IV fracture. MRI, as described earlier, is a more sensitive method for early diagnosis of ON. The reactive interface between necrotic and viable bone in traumatic ONF can be detected by MRI. We

performed a prospective study in patients with transcervical fractures of the femur and found a high incidence of MRI abnormalities indicating ONF (8/17 patients) by one month after internal fixation of fracture.²⁹ Animal models of traumatically produced ON can also be used to identify useful diagnostic and therapeutic methods for ON if they are highly reproducible.

A. GOAT MODEL

In 1910, Legg, Calve, and Perthes individually described a disease (Legg-Calve-Perthes disease) manifesting as deformation of the developing hip in children. The exact etiology of this disease has not been determined, although extensive clinical, radiographic, and animal studies of it have been performed. None of the animal models developed has been able to reproduce the variety of lesions seen in children. The growth pattern resulting in the most severe femoral head deformity is one in which anterolateral physeal growth is arrested while perichondral ring growth and posteromedial epiphyseal growth continues or accelerates. An animal model that mimics this growth pattern and the resultant morphology of the affected femoral head is needed. Newton et al. reported a surgical method for creating morphologic changes similar to those observed in Legg-Calve-Perthes disease in an animal model.³⁰

1. Materials and Methods

Two groups of animals were used in the experiment. Four mixed-breed goats between one and three months of age underwent surgery to develop the surgical procedure described below and were followed for approximately two months. The objective of the surgery was to ablate the physis anterolaterally while leaving as much of the perichondrial ring intact as possible. Another group of 10 pure-bred Alpine goats three months of age also underwent surgery using the same procedure and were followed radiographically for approximately 13 months. Under general anesthesia with halothane and oxygen, an anterolateral approach was used to expose the hip joint. To access the physis, a window approximately 2×5 mm in size was curetted from the articular cartilage over the physis. The electrocautery blade was introduced into the physis through this window to a depth of approximately 2 cm across the width of the growth plate. The blade was inserted into the physis three to four different times in a fan pattern and coagulated.

The first group of animals was sacrificed two months postoperatively, and the harvested femurs were fixed in neutral-buffered formalin. Measurement of the total length of the femur, the transverse and vertical diameters of the femoral head, and the distance across the femoral neck were made for the operated and control femurs. After proper fixation, the femoral head sections were decalcified, processed, and embedded in paraffin. The paraffin blocks were sectioned, and stained with hematoxylin and eosin. The extent and location of the growth plate by fibrous tissue, fibrocartilage, and bone bridges were evaluated quantitatively using planar morphometry. The anteroposterior radiographs were scanned using a X ray scanner. The radiographic images were enhanced using Adobe Photoshop (Adobe Systems), and a radiographic template was created using Aldus Freehand (Aldus, Seattle, WA, USA).

2. Essential Findings

In each case the test femur was shorter than the control femur ($p < 0.05$), suggesting that longitudinal growth of the femur was partially arrested. Measurements of the remaining parameters for the test femurs were not significantly different from those for the control femurs. The gross observations indicated that the physis, and its potential for complete longitudinal growth, were affected by the surgical procedure. In test specimens, the physis exhibited irregular alignment of the growth plate chondrocytes compared with that in controls. Variable thickness of the growth plate, including the cartilage tongues from the physis in both specimens, may have resulted from localized avascularity of both the metaphysis and the epiphysis. Bone bridges, fibrous tissue, and

fibrocartilage had replaced the physis. The histological findings following surgery were comparable to those reported for patients with Legg-Calve-Perthes disease. Quantitative histologic evaluation showed that the physis was affected in each femoral head, although the area of disturbance of the physis differed among the specimens. The overlays produced using graphic analysis showed the changes in growth of the proximal femur. The surgical procedure was effective in ablating the capital femoral physis, and induced changes in the growth of the femoral head. However, the resultant deformities did not mimic the changes identified in the graphic analysis study, perhaps because of inconsistencies in the surgical techniques used for ablation, which will require further modification.

B. CANINE MODEL BY DEEP FREEZING

1. Introduction

New treatment options for the management of ON have been proposed, such as electromagnetic stimulation and vascularized bone grafting procedures. However, the process of bone repair resulting from clinical use of these treatment modalities has not been clearly defined, since material for evaluation and histologic analysis usually is obtained after treatment has failed. An experimental model is thus needed to evaluate these different therapeutic modalities. Malizos et al. investigated the efficacy of a surgically invasive technique, consisting of femoral head dislocation, soft-tissue stripping, and deep freezing, in the induction of ON in dogs, and also characterized the process of spontaneous bone repair within such lesions.³¹

2. Materials and Methods

Twenty-six mature beagles were used, four of which were evaluated in a pilot study. Dynamic bone remodeling in beagles is similar to that in humans. Under general anesthesia with halothane, the right hip was approached through a posterolateral incision. After dislocation of the femoral head, a surgical rubber tube was coiled around the femoral neck and liquid nitrogen was circulated through the tube for three min. Two animals died during the operation.

After sacrifice, the proximal third of both femurs was prepared for mineralized bone sections. Tissue fixation was performed in 70% alcohol for 48 hours. The samples were then embedded in methylmethacrylate and cut with a heavy duty microtome into complete sections in a frontal plane. Serial cuts from the central portion of the specimens were obtained for staining with Goldner trichrome and toluidine blue. Histomorphometric analysis was performed using a semiautomatic computerized system (OsteoMeasure, Osteometrics, Atlanta). To study the kinetics of bone healing, three different bone-labeling fluorochromes were administered intravenously for two consecutive days. Two dogs were killed after one week postoperatively, and two others, after two weeks. Four animals each were killed at 4, 8, 16, and 24 weeks postoperatively. At necropsy, both femurs were obtained and radiographed prior to the tissue preparation.

3. Essential Findings

A mixture of cellular and erythrocyte debris was observed in specimens examined two hours after the freezing procedure. By two weeks after the induction of ON, histologic changes in bone were more uniform. The osteocytic lacunae were empty or contained eccentrically located osteocytes. The marrow cavities contained amorphous debris, and mineralization was present on the surface of the trabeculae. One month postoperatively, soft-tissue debris was no longer present in the marrow cavities, and the repair process had already been established. Spontaneous healing originated mainly from the adjacent viable bone by migration of undifferentiated mesenchymal tissue into the necrotic bone, fibrosis, and, finally, formation of new bone. Planimetric measurement showed that at four weeks after the induction of necrosis the mesenchymal reparative tissue,

occupied $13.1 \pm 4.1\%$ of the total area of initial necrosis, and the zone of osteogenesis occupied $4.9 \pm 2\%$ of this area. At eight weeks, the mesenchymal reparative tissue occupied $15.8 \pm 3.3\%$ and neo-osteogenesis, $6.9 \pm 3.5\%$ of the initially necrotic bone. At 16 weeks, the corresponding percentages were $55.3 \pm 7.7\%$ and $20.1 \pm 8\%$, and at 24 weeks, $65.3 \pm 16.5\%$ and $28.2 \pm 8.8\%$. Microscopic examination under fluorescent light revealed complete absence of fluorochrome fixation in the area of necrosis, confirming the lack of bone viability. In the 4-week specimens, labels were present in the form of patchy tallow fluorescent spots extending throughout the area of neo-osteogenesis. The rate of bone formation per unit area of surface was $64.1 \pm 16.1 \mu\text{m}^3/\mu\text{m}^2/\text{yr}$, which exceeded the average formation ($42.5 \mu\text{m}^3/\mu\text{m}^2/\text{yr}$) in cancellous bone at distant sites in normal mature beagles. Radiographic changes became evident only four weeks after the induction of ON, and began as a band of radiodensity across the medulla at the margin of the necrotic area. More proximally, an extensive zone of marked osteopenia was present, including spots of complete trabecular loss. At 16 weeks, the areas with osteopenia and lysis in the trabecular bone had advanced further proximally near the femoral neck and at the greater trochanter. There was no loss of the contour of the articular surface at any of the time points of examination.

C. CANINE MODEL BY DISLOCATION AND VESSEL LIGATION

1. Introduction

The etiology of femoral head necrosis following traumatic hip dislocation remains obscure, in large part because no suitable models have been established for the study of its pathophysiology. Microangiographic studies of the distribution and anastomosis of arteries supplying the femoral head may account for the difficulties in animal experiments to induce ON when the femoral head is not manipulated by unrealistic methods and the marrow cavity is preserved. Nishino et al. recently reported that they established a new model of ON by dislocating the hip joint and ligating the medial and lateral circumflex femoral arteries and veins.³²

2. Materials and Methods

Mongrel adult dogs each weighing 8–12 kg were used. The dogs were divided into three groups, which were subjected to hip dislocation alone, ligation of blood vessels alone, or both hip dislocation and ligation of blood vessels. At two and four weeks after treatment, five animals in the hip dislocation only and ligation only groups, and 10 animals in the dislocation plus ligation group were killed, and pathologic studies were performed. In addition, MR images of the model were evaluated for the combined dislocation and ligation group. Femoral head blood flow was measured in 10 dogs to evaluate quantitatively to what extent dislocation and blood vessel ligation affected femoral head blood flow volume.

After the induction of anesthesia by ketamine, sodium pentobarbital (5mg/kg/h) was administered intraperitoneally with blood pressure monitoring to maintain general anesthesia. An approximately 1-cm-long incision was then made on the capsule through a lateral approach, the round ligament was served, and a cervical vertebra spreader used to create and maintain the dislocation. The hip was dislocated posteriorly, and the extent of the dislocation was maintained constant at a distance of one and a half femoral heads from the original position for nine hours. In the ligation group, the lateral and medial circumflex femoral arteries and veins leading to the femoral neck were ligated and cut.

The excised femoral heads were sliced parallel to the coronal plane and stained with hematoxylin and eosin for pathologic examination. In order to evaluate the effect of dislocation and blood vessel ligation on femoral head blood flow, femoral head blood flow volume was measured in 10 adult dogs at room temperature ($20\text{--}22^\circ\text{C}$) using the electrochemically generated hydrogen clearance method. Measurements were made using the method of Stosseck et al.,³³ with the center of

the femoral head selected as the site of measurement. MR images of excised femoral heads were examined within two hours after excision. MRI was performed using a Sigma 1.5-Tesla superconducting magnet. The coronal plane was used for all examinations.

3. Essential Findings

In the dislocation only group, no necrosis was observed in any of the 10 animals at either two or four weeks, although some cases exhibited congestive changes and a decrease in the number of marrow cells. In the ligation only group as well, no empty lacunae were found at either two or four weeks, and no case exhibited findings of ON. In the combined dislocation and ligation group, ON was observed at two weeks in eight of 10 (80%) and at four weeks in eight of 10 (80%) dogs. Also, in the majority of cases, appositional bone formation, which is thought to be a manifestation of the repair process of ON, was observed. The distribution of the necrotic area was similar to that for human femoral head necrosis, with necrosis more extensive on the joint side than on the neck side, and clearer manifestations of repair on the neck side. Although the extent of ON exhibited considerable individual variation, in most cases ON was found over a wide area centering on the weight-bearing region. The rate of femoral head blood flow in the control group prior to dislocation was 78.8 ± 19.7 ml/min/100/ml. In the dislocation only group it was 32.1 ± 15.4 ml/min/100ml, dropping to 40.7% of the predislocation value. In the dislocation plus ligation group, it was 11.6 ± 10.3 ml/min/100/ml, representing a marked decrease to 14.7% of the predislocation value.

D. SWINE MODEL

1. Introduction

MRI is the diagnostic modality most useful for the early detection of femoral head necrosis. However, a negative MRI does not exclude the diagnosis of ON. In the setting of acute trauma, there have been few findings regarding the interval of time during which MRI reliably exhibit findings consistent with ON. Seiler et al. examined the correlation of the time sequence of MRI changes to changes with femoral head blood flow and the ultimate appearance of femoral heads in a miniature swine model of posttraumatic proximal femoral ON.³⁴

2. Materials and Methods

Twelve 40–60 kg skeletally mature (age 14–20 months) Gottinger miniature swine were used. One animal died perioperatively from the anesthetic, leaving 11 swine available for study. Under general anesthesia with a mixture of halothane and oxygen, a posterolateral approach to the right hip was used for all animals. A 5.0 mm hollow aluminum screw with a Teflon coating and plastic sleeve assembly was inserted into the posterior wall of the acetabulum. The femoral head blood cell flux (BCF) was measured through the hollow screw and sleeve assembly with a 2.2 mm flexible laser Doppler flowmetry probe. A basilar femoral neck osteotomy (simulating a fracture) was then created with a 1.5 mm osteotome, and the BCF was again measured. The fracture was reduced under direct vision and internally fixed with two 1.6 mm commercially pure titanium Kirschner wires.

Repeated femoral head BCF values were obtained at one, two, four, and eight weeks postoperatively. At the time of the index operation, the end of the screw and sheath assembly were placed in subcutaneous tissue to permit subsequent access for percutaneous Doppler assessment. Doppler measurements were then obtained and recorded as previously described. All magnetic resonance images were obtained on a 1.5 Tesla imager. MRI intensity ratios were computed as the ratio of the intensity of the experimental hip divided by the intensity of the hip on the control side. Both femurs in each animal were explanted after sacrifice and immediately stored at -70°C . The femoral

heads were sectioned and examined by light and fluoroscopic microscopy. The specimen was graded as having no (0), intermediate (1) or significant (2) involvement with each of the following seven histologic parameters associated with ON: (1) empty osteocyte lacunae; (2) marrow invasion with mesenchymal cells; (3) vascular ingrowth/ hypertrophy; (4) creeping substitution; (5) marrow fibrosis; (6) decrease in subchondral bone thickness; and (7) chondral collapse. Maximum total ON score was 14 points, with higher scores representing more extensive femoral head ON. Plain radiographs of all explanted femurs were obtained. Bone density data were calculated by a video image analyzer linked to image processing software via a Macintosh computer. Using this system, the trabecular bone surface area could be expressed as a percentage of a given field of view in the suprolateral aspect of the femoral head.

3. Essential Findings

Significant inter-animal variation in femoral head blood cell flux, as assessed by Doppler, was observed. The mean baseline value was 1490 ± 465 mV. After a femoral neck fracture, this value decreased to 869 ± 211 mV. Significant decreases in femoral head blood cell flux were observed at week 1, 4, and 8. At week 8, BCF averaged 262 ± 83 mV. Femoral head blood flow decreased immediately after fracture and continued to diminish with time. Seven animals had some degree of failure of internal fixation with loss of anatomic reduction by one week postoperatively, which was confirmed visually at the time of final exploration. In four animals, fixation remained intact throughout the period of observation and this group was analyzed separately. MRI signal intensities in the femoral head (MRI intensity ratio) at four and eight weeks were significantly less when fixation failed than when it was intact. ON histology grades averaged 7.6 ± 0.8 on the experimental side and 1.6 ± 0.4 on the control side ($p < 0.01$). The range of histology grades was 0.0–4.5 for the control hip and 4.3–11.2 for the experimental hip. There was no significant difference between the intact fixation group (6.5 ± 1.0) and failed fixation group (8.4 ± 1.0) in histology grade. The mean bone density on the experimental side was 49%, while that on the control side was 56% ($p < 0.01$). Histologic grade, bone density value and blood flow values were each unrelated to changes in MRI signal intensity.

IV. APPLICATIONS OF ANIMAL MODELS

Although many other experimental animal models of ON have been reported, only several recent, reproducible models are introduced in this chapter. The models described here can be used for various purposes. Nontraumatic models are more suitable for evaluation of the etiology of ON than traumatic models, the procedures in which are too radical. The pathology of ON could be clarified with different approaches to investigate the specimens much earlier than ON occurred.

Similarly, nontraumatic models may be more appropriate for use in identifying drugs or materials useful for preventing the genesis and/or progression of ON. Administration of those agents before the final procedure to induce nontraumatic ON is performed may reveal new therapeutic modalities for ON.

Nontraumatic and traumatic models are equally useful for development of new surgical approaches for ON. Clinical ON of the femoral head (ONF) is thought to follow a defined course once it has developed in the subchondral area of the femoral head. For both nontraumatic and traumatic ONF, the prognosis largely depends on the location and size of lesions. Regardless of the cause, continued weight-bearing on devitalized bone leads to collapse, joint deformity, degenerative change, and painful dysfunction in patients with relatively large areas of necrosis. In this regard, traumatic models may be more useful if the size and location of ON can be controlled well with specific surgical procedure.

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15 Animal Models of Osteopenia or Osteoporosis

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I. INTRODUCTION

Much of the progress during the last one-sixth of the twentieth century in prevention and treatment of osteoporosis is attributable to today's excellent animal models. The purpose of this chapter is to describe the current status of animal models that address osteoporosis and osteopenia and then recommend strategies for applying them.

US Food and Drug Administration guidelines for using animals in preclinical tests of agents intended to treat osteoporosis¹ recommend animals either losing bone or have ovariectomy (OVX)-related osteopenia. Histologic, densitometric, biochemical, and biomechanical data from both the OVX rat and a larger species with Haversian remodeling are required.

Full parallelism of human symptoms with any single *in vivo* animal model does not exist. Thus, a strategy emphasizing small animals with limited use of a large animal is recommended. These criteria place the highest value on animal models that match the clinically apparent behaviors of osteoporosis and osteopenia. Models that give inconsistent results in the hands of numerous investigators, involve convoluted conditions, or require difficult species, win low marks. We find using relevant methods in an adult animal to produce consistent partial symptoms better than using less physiologic circumstances to develop a full set of symptoms.

II. GENERAL CONSIDERATION

A. THE CRITERIA

Animal models for the adult skeleton have been reviewed elsewhere.²⁻⁸ This chapter not only updates previous points, but also raises new issues. Its approach is to: (1) highlight critical data that can be assessed *in vivo* in humans and (2) show how today's animal models match those data.

An animal model of osteoporosis should have lengthy growing and adult skeletal phases. Peak bone mass in women occurs between ages 25–50 yrs, and is important because of its likely role in the late-life development of osteoporosis.⁹⁻¹⁰ A bone mass measurement at age 50 is the best predictor of future fracture in healthy persons.¹¹⁻¹² Peak bone mass is precisely measurable and 80% heritable,¹³⁻¹⁴ making it a good phenotype for quantitative trait linkage studies.¹⁵

Humans not only have a menarche and regular, frequent ovulatory cycles, but also experience bone loss at cessation of ovarian function. This is best shown by osteopenia in amenorrheic individuals,¹⁶⁻¹⁸ the bone accumulation that occurs upon resumption of normal menses,¹⁸⁻¹⁹ and the identification of oligomenorrhea/amenorrhea and late menarche as osteopenia risk factors.²⁰ Only mammals (and humans) with regular, frequent ovulatory cycles and high peaks of 17-estradiol (E2) may suffer estrogen-depletion bone loss. Regularly cycling female mammals accumulate an estrogen-related component of bone that integrates into the skeleton²¹ and disappears at menopause. Animals with infrequent cycles and low E2 peaks might develop too small an estrogen-related bone compartment to allow estrogen depletion bone loss. Over two-thirds of women experience natural menopause. Of the rest, many have preserved ovarian function or prompt estrogen replacement therapy (ERT). Most animal skeletal models of estrogen-depletion employ surgical OVX.^{22,23} No meaningful differences in bone behavior between surgical and natural menopause exist.²⁴

B. BONE LOSS AND TURNOVER RATE RISE AFTER ESTROGEN DEPLETION

Following estrogen-depletion, bone loss accelerates in cancellous regions^{25–26} and at endocortical surfaces^{27–28} in multiple sites,²⁹ then decelerates into a semi-plateau phase.³⁰ Estrogen-depletion changes in Haversian remodeling are poorly documented. Cancellous and endocortical bone loss is accompanied by increased turnover^{31–32} and a marked, transient negative calcium balance.³³ Histomorphometric signs of increased transmenopausal turnover are readily shown within individual humans.³⁴ Oophorectomized or menopausal women given estrogen replacement (ERT) show a smaller bone turnover rise,^{31,35} less bone loss,^{36–37} and fewer fractures than those given no ERT.^{36,38} This ERT response is demonstrable by histomorphometric techniques in humans and animals.^{35,39,40} All these behaviors should not only exist in an accurate animal model of the menopause, but be measurable by similar techniques.

C. OSTEOPOROTIC FRACTURES AND STEADY STATE OSTEOPENIA

Because of today's excellent ability to quantitate bone mass,⁴¹ the World Health Organization endorses a numeric/symptomatic definition of osteoporosis, as all women with bone mass at one or more bone sites 2.5 standard deviations below the young adult normal, or with a history of low trauma fractures of the spine, hip, or wrist.⁴² An animal model that develops fragility fractures after estrogen depletion would facilitate pre-clinical evaluation of agents' anti-fracture efficacy. However, today's excellent animal models of estrogen-depletion bone loss exhibit no low trauma fractures of critical bone sites.^{43–44} The animals, unlike humans, may lack the contribution of low peak bone mass necessary to put them below their fracture threshold. One animal model with low peak bone mass, the SAM/P6 mouse,⁴⁵ has fragility fractures.⁴⁶

D. REMODELING

Remodeling, the *in situ* removal and replacement of old bone tissue by new bone tissue, is the dominant process in the adult human skeleton.⁴⁷ Cancellous bone surfaces, including endocortical surfaces, experience the highest remodeling rates in the skeleton.²⁸ Mild, long-standing deficits in remodeling may cause cancellous osteopenia, a risk factor for fragility fractures.

Cortical bone plays a dominant role in skeletal strength. Adult humans have Haversian (intracortical) bone remodeling. Though the effect of estrogen depletion on Haversian remodeling is not well known,⁴⁸ post-menopausal osteoporosis is characterized by only minimal cortical porosity. Nonetheless, an accurate animal model should display Haversian remodeling because of its importance in the maintenance of cortical bone strength. Furthermore, first generation anti-osteoporosis agents tend to suppress remodeling. The inability to obtain cortical bone biopsy specimens from humans in Phase III/IV trials means that Haversian remodeling cannot be studied in humans. Therefore, adverse remodeling changes caused by anti-osteoporosis agents are best revealed in studies of animals with Haversian remodeling. An accurate animal model would thus display such activity in its skeleton.

E. TIMEFRAME COMPRESSION

The time from peak bone mass attainment until the development of fragility fractures is 30+ yrs. In women, accelerated estrogen-depletion bone loss lasts 5–8 yrs. An effective animal model known to experience peak bone mass and post-OVX bone loss should decrease both times by an order of magnitude. Convenience for animal models is denominated as cost of purchase, availability, housing, handling difficulties; and designing/implementing/validating new analysis procedures. Having validated small animal models is the best route to including the largest number of investigators in any research field. Using a highly accurate animal model occasionally can be so inconvenient that it may be more difficult than a human study.

III. ANIMAL MODELS FOR OSTEOPOROSIS

Animals that should receive major consideration in osteoporosis research are mouse, rat, dog, pig, sheep, and nonhuman primate. Because the above criteria are directed at clinical observations in humans, the animal models will be evaluated as to how they duplicate those outcomes (Table 1).

A. MOUSE

1. In General

The mouse is now rising as an *in vivo* model for osteoporosis research. It is the ideal model of osteopetrosis,^{49–50} osteoclast and stromal cell ontogeny,⁵¹ and cytokine and marrow studies.^{52–54} It will become even more popular for the ease with which its genome can be manipulated.^{55–58} Mice have been used to identify and characterize osteopetrosis genes,⁵⁰ both with linkage and transgenic animal studies. The disclosure of genes associated with osteopetrosis, a disease of osteoclast dysfunction, may lead to the discovery of agents that inactivate osteoclasts. Strains of mice with low and high peak bone mass⁵⁹ lend themselves to genetic investigations.¹⁵ Considering the availability of SAM/P6, C57BL/6J, and C3H/HeJ,⁵⁹ proper breeding techniques with probing the whole genome for high density polymorphic markers should identify one or more genetic loci linked to bone mass in mice.

Estrogen has similar effects on the mouse and human skeletons. Cancellous,^{58,60} but not cortical⁶¹ bone loss occurs soon after OVX in the distal femur of strains like Swiss-Webster. Cancellous and cortical bone loss in the vertebrae and femur occurs after age one yr.^{62–63} Hypogonadal female mice are osteopenic.⁶⁴ ERT at doses of ~10 mg/kg/d 2–3X/wk prevents estrogen-depletion bone loss.⁶⁰ Increased bone formation and woven bone deposition after E2 administration occurs at doses above 50 mg/kg/d,^{60–61,65} a response never seen in humans. The dose-related E2 effects in the mouse do not stop the consideration of certain strains of mice as models of the estrogen-deplete human skeleton.

The ability to construct designer animal models (i.e., transgenic mice) is now driving mouse skeletal experiments. Validation of the mouse as an animal model for osteoporosis calls for targeted experimental work to document fully its skeletal response to E2. The time course and site specificity of estrogen-depletion osteopenia must be established in a strain specific fashion as it has been for the rat.^{22,66} The bone response to OVX in strains that are the basis of transgenic models must be established.

2. Practical Problems

Specimens adequate for histomorphometric study of cancellous bone mass, structure, and turnover can be obtained from the distal femur. Mouse bones contain such small amounts of mineral that conventional dual energy X ray absorptiometry (DXA) cannot be used. Modified DXA (slower speed, smaller collimator) is the best alternative.⁶⁷ Equipment like pQCT (peripheral quantitative computed tomography) and peripheral DXA (pDXA) is useful,⁵⁹ but not yet widely applied.

3. The SAM Mouse

The SAM/P6 (senescence accelerated mouse) mouse has low peak bone mass and late-life fractures.^{45–46,68} It is the only experimental animal with both low peak bone mass and fragility fractures of aging. The SAM mouse needs full genetic,⁶⁹ hormonal,⁷⁰ and biomechanical characterization, including site specificity for fractures. If it lacks collagen defects seen in osteogenesis imperfecta^{55,71} it may, when combined with osteopenia prevention approaches like ERT or bisphosphonates, allow study of the role of low peak bone mass in late-life fractures. It will definitely provide chances to identify genes contributing to peak bone mass.

TABLE 1
Summary of *In Vivo* Animal Models for Osteoporosis

Attribute	Human	Avian	Mouse	Rat	Dog	Pig	Sheep	Primate
Growth and Adult Phases?	Yes	OK	OK	Yes	Yes	Yes	Yes	Yes
Menstrual/Estrus Cyclicity/Natural Menopause	28d/Yes	daily/No	inducible/ Yes	4–5d/Yes	205d/No	21d/?	21d seasonal	21–28d/Yes
Bone Loss after Estrogen Depletion	Yes	?	Yes	Yes	Not Consistent	Weak	Not Consistent	Yes
Response to Estrogen	Turnover	Formation	Turnover (dose-related)	Turnover	Not Consistent	?	?	Turnover
Development of Osteoporotic Fractures	Yes	No	No	No+	No+	?	?	No+
Cancellous Remodeling	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Haversian Remodeling	Yes (study site difficult)	No	No	Little; inducible	Yes	Yes	Yes	Yes
Timeframe Compression	No	?	Yes	Yes	No	Some	Some	Some
Convenience	OK	Yes	Yes	Yes	Weak	Yes	Yes	Yes
Cost Effectiveness	Yes	No	Yes	Yes	Weak	?	?	Yes

B. RAT

1. In General

The rat has long provided data about skeletal behavior. It gave the first evidence that osteoclasts ingest bone mineral⁷² and early evidence about the hematogenous origin of osteoclasts.^{73–74} The rat skeleton was once held unsuitable as a human skeletal model because many growth cartilages in male rats remain open past age 30 months.⁷⁵ Recent studies prove that growth cartilages at important sampling sites close by age 6–8 months in female rats, much earlier than males.^{75–78} Those once holding reservations about the rat as an adult skeletal model because of its “continuous growth and lack of remodeling,” have relented. They now only caution investigators to use female rats of age 6–9 months and avoid studying Haversian remodeling.⁷⁹ Periosteal expansion continues until about age 10 months, the age of peak bone mass in the female rat.⁸⁰ The mean healthy lifespan for rats is 18–21 months, but can be extended by OVX that prevents estrogen-dependent mammary tumors and restrictive feeding,⁸¹ improving the possibility of doing FDA-mandated skeletal studies in rats.

Adult female rats have a regular estrus cycle with E2 levels spiking every four days.⁸² After age one yr, the fraction of rats in constant diestrus rises gradually,⁸³ and cancellous bone loss frequently occurs. Spikes in E2 end as bone loss occurs, suggesting a similarity to human menopause. Following OVX, site-specific loss of cancellous bone mass and strength occurs, accompanied by increased bone turnover,^{66,84–85} and decelerates into a plateau phase.^{22,43–44,66} These features mimic well the bone changes following OVX or menopause in humans. Not all cancellous bone sites in the rat show such bone loss,⁸⁶ tightening the parallel of the rat and human skeletons. DXA, the current state-of-the-art for measuring bone mass in humans, is readily applied in rats.⁸⁷

The OVX rat is a textbook example of a bench scientist introducing a highly relevant, widely-applied pre-clinical animal model.^{22,40} OVX rats given prompt ERT show neither a turnover rise,^{39–40,88} nor bone loss.^{40,88–89} Bisphosphonates,^{90–91} calcitonin,^{92–93} and selective estrogen receptor modulators^{94–95} also block the rise in turnover and bone loss in OVX rats, just like in humans.^{96–97} Though one group suggests high dose E2 stimulates bone formation,^{98–99} these data have been discounted by others using similar experimental designs who correctly consider the use of growing rats and a long fluorochrome labeling interval.¹⁰⁰

The rat, like most osteoporosis animal models, lacks fragility fractures related to osteopenia. This problem has been overcome by mechanical testing of the vertebral body,^{101–102} femoral shaft,¹⁰³ and proximal femur.^{102,104} Using vertebral body strength as a surrogate for human vertebral body fracture likelihood may be a more direct test of pre-clinical anti-fracture efficacy of anti-osteoporotic agents than bone mass measurement. Such preclinical testing could avoid the problems observed with sodium fluoride as an osteoporosis treatment.^{105–106} The recent development of *ex vivo* tests of bone fragility in small and large animals that now serve as surrogates for osteoporotic fracture is a similar example.^{101–102,104,107}

Adult rats have adequate cancellous bone remodeling to permit useful experiments.^{79,108–109} The relative amount of modeling and remodeling activity appears related to age. In the proximal tibial metaphysis, reversal lines at the base of cancellous osteons are mostly absent in four month old rats,⁹⁸ but present in cancellous osteons of 9–12 month old rats,¹¹⁰ suggesting that remodeling activity increases with age in the rat. In most of its cortical bone, the rat has near zero levels of Haversian remodeling. However, processes resembling intracortical remodeling are induced by anabolic agents¹¹¹ or stressful metabolic conditions.^{112–113} The rat has such low levels of Haversian remodeling that it cannot be used for studying Haversian remodeling, especially when evaluating agents that suppress remodeling. The female rat reaches peak bone mass by age 10 months, a thirty-fold timeframe compression when compared to the adult human. In three month old OVX rats, the phase of accelerated estrogen-depletion bone loss lasts three–four months in the proximal tibial metaphysis,²² a 20-fold time saving over estrogen deplete women. The rat is among the most convenient of experiment animals to handle and house.

2. Specific Recommendations for Rat Experiments

Food intake in OVX rats should be restricted to block OVX-induced hyperphagia. It may involve matching intake to sham rats (~10% restriction), restricting intake to match weights of OVX rats to sham rats (~20% restriction), or caloric restriction (~30% restriction).⁸¹ All types of food restriction accentuate bone loss in OVX rats,^{114–115} creating more reliable cortical bone loss and quickening the onset of estrogen-depletion cancellous osteopenia.

Treatments that may prevent OVX-induced bone loss should start immediately after OVX. New techniques of bone structural study show that bone changes begin before 10 days post-OVX.^{116,117} Delaying preventive treatment decreases the chance that a test agent can have its optimal effect, analogous to using an antibiotic in curative vs. preventive mode. Once the estrogen depletion bone loss process has begun, it may be difficult to stop.

3. Summary

The OVX rat duplicates the most important clinical features of the estrogen-deplete adult human skeleton. Its site-specific cancellous osteopenia is among the most prompt, certain physiologic responses in skeletal research. Ample time exists for experimental designs that either prevent estrogen-depletion bone loss or restore bone lost after estrogen depletion, particularly when using restrictive feeding. Its response to ERT parallels the human. The rat's low levels of Haversian remodeling present no problem for testing agents that may prevent loss of, or rebuild lost cancellous bone. Its lack of fragility fractures is overcome by biomechanical testing. Rats are convenient; unbred females aged 6–10 months are best for treatment phases of experiments. Densitometry, biochemistry, histomorphometry, and mechanical testing are used readily.

C. AVIAN, GUINEA PIG, RABBIT, FERRET, AND CAT

Adult female birds have a daily egg-laying cycle that corresponds to alternating deposition and resorption of medullary bone.¹¹⁸ Bone accumulation occurs with rising serum E₂; removal accompanies falling E₂. E₂ treatment of male birds causes medullary bone deposition.¹¹⁹ While hypoestrogenemia in birds is associated with medullary bone loss, just as estrogen depletion in mammals is associated with osteopenia, the course of avian bone mass following OVX is unknown. Current data suggest that birds have little remodeling. The E₂-related bone buildup may one day aid in understanding peak bone mass accumulation in pubertal females,^{21,120–121} but will hinder experiments about osteoporosis, an adult human disease, because it has no counterpart in adult mammalian physiology. Birds are irrelevant for osteoporosis research because their skeletal behavior does not match known features of adult human osteoporosis.

Though guinea pigs, rabbits, ferrets, and cats have been used in osteoporosis research,^{122–124} too few published experiments exist to assess model validity. OVX guinea pigs do not lose bone.¹²³ Adult (8 month old) rabbits might serve as a model for Haversian remodeling. The ferret has Haversian remodeling,¹²⁵ but its normal skeletal physiology, including the accumulation of estrogen dependent bone that accompanies normal cyclicity in other mammals, is light cycle dependent.¹²⁶

D. Dog

The adult dog is generally a reliable model of the adult human skeleton. Haversian and cancellous osteons remodel as in humans, though more rapidly.¹²⁷ Skeletal findings in the adult dog parallel the adult human for corticosteroids,^{128,129} uremia,¹³⁰ bisphosphonates,¹³¹ and PTH excess.¹³² In contrast to all other uses for the adult dog as a model of the adult human skeleton, the OVX dog is problematic. Many individual studies lack significance, but the data in bulk¹³³ suggest that 8–10% annual bone loss occurs in newly-OVX dogs. Most studies indicate a minor transient post-OVX rise in bone formation, but some¹³⁴ suggest that formation falls rapidly by 50%,

without a transient increase. The latter suggests a dissimilarity to findings in transmenopausal humans and other animals, where the transient turnover rise³⁴ is well known.³¹ ERT tends to suppress turnover, but with uncertain effects on bone mass.¹³⁵ E2 levels in the dog are usually very low, rising twice yearly.¹³⁶ E2 spikes in rats for 18 hours every four d⁸² and monthly in women.¹³⁷ The estrus cycle in monkeys is similar to humans, but E2 peaks only about half as high.¹³⁸ Dogs' integrated E2 exposure, only marginally less than in rats, is only one-fourth that in humans and similar to primates, except for the peaks. These differences could cause the dog to develop only a small estrogen-dependent cancellous bone compartment.

The adult dog, with its Haversian remodeling, is an excellent model of the adult human skeleton except for its response to estrogen depletion. Though the OVX dog probably has estrogen-depletion osteopenia, the poor inter-laboratory reproducibility for this finding has caused most investigators to abandon it. The FDA guidelines advise not using the OVX dog.¹ The main problem is that most studies have insufficient power (N=6-9/group) to detect 8-10% bone loss.¹³³ Despite its inconsistent estrogen depletion bone loss, the dog is fine for testing the Haversian remodeling effects of agents with anabolic effects on cancellous bone. A strategy using one large animal for both Haversian remodeling and estrogen-depletion bone loss makes sense.

E. PIGS AND SHEEP

The OVX pig has been disappointing, showing only minor structural deterioration and bone loss.¹³⁹⁻¹⁴¹ Its regular estrus cycle is shorter than in the human. Pigs, with Haversian remodeling, have been used to study skeletal effects of bisphosphonates, fluoride, and exercise.¹⁴²⁻¹⁴⁴ Peak bone mass occurs after age 3, confounding experimental efforts by introducing cost difficulties. More work is necessary for the pig to gain acceptance as a large animal model of estrogen depletion bone loss.

Ewes have a regular estrus cycle during fall and winter, but show anestrus during longer days.¹⁴⁵ Post-OVX skeletal behavior is less consistent than in post-menopausal women, rats, or monkeys.¹⁴⁶ Bone biomarkers indicate accelerated remodeling by three months post-OVX;¹⁴⁷ marginal relative osteopenia occurs at a few bone sites by 6-12 months post-OVX.¹⁴⁸ Fluoride and glucocorticoid effects in ewes parallel findings in other animals and humans.^{122,129,149-151} Aged sheep pose little problem for handling. Existing data thus reveal inconsistent OVX-related bone loss as in pigs. New data that assess the age of peak bone mass and more data on post-OVX bone loss are needed to validate the adult ewe as a model of osteoporosis.

F. NONHUMAN PRIMATE

Peak bone mass occurs at age 9-11 yrs in cynomolgous and rhesus monkeys and baboons.¹⁵²⁻¹⁵⁴ Nonhuman primates have a 28 day menstrual cycle and experience natural menopause at age 18-20 yrs. Pedigree studies of nonhuman primates can establish genetic linkage to bone mass.^{15,155} Combining genetic studies of the mouse and nonhuman primate is a multi-species approach to identifying genes that control peak bone mass in humans.

Nonhuman primates (NHP) show decreased bone mass and strength with increased turnover after OVX¹⁵⁶⁻¹⁵⁹ or GnRH agonist treatment.²³ Absolute bone loss of ~5% occurs by three months post-OVX and plateaus at 8% by nine months post-OVX.¹⁶⁰ Though the ERT response has not been studied, bisphosphonates prevent post-OVX bone loss.¹⁶⁰ Adult NHPs show bone loss with age.¹⁶¹⁻¹⁶² Post-OVX bone changes are frequently masked by using animals still gaining peak bone mass.^{157,162} Histomorphometric studies of estrogen-depletion cancellous bone loss in NHPs and humans not only yield similar values,¹⁶³⁻¹⁶⁵ but also allow studies of Haversian remodeling shortly after OVX.⁴⁸ Late life spinal pathology in baboons¹⁶⁶⁻¹⁶⁷ and rhesus monkeys¹⁶⁸ is osteoarthritis, not osteoporosis. Baboons experience osteopenia¹⁶¹ with an age-related decline in anterior vertebral height like that in osteoarthritis rather than osteoporosis;¹⁶⁶⁻¹⁶⁸ no such decline is seen in rhesus.¹⁵⁴ Thus, NHPs

may not be a model of fragility fractures, because osteoarthritis and osteoporosis tend to be mutually exclusive.¹⁶⁹ The interference of osteoarthritis in older primates with spine DXA should also be considered.¹⁷⁰

NHPs have cancellous and Haversian remodeling comparable to humans. Animals aged 9–11 yrs, the age of peak bone mass, should be used. Bone loss after OVX lasts nine months, a six–eight-fold timeframe improvement over humans. NHPs are today's large animal model of choice for adult skeletal research when information about Haversian remodeling is required.

G. SUMMARY

The 10 month old female rat is now the animal of choice for studies of every feature of human osteoporosis except Haversian remodeling. It has reached peak bone mass and can be manipulated through OVX to simulate clinical osteoporosis in adult women. Routine methods for humans, like histomorphometry, DXA, and, serum biochemistry work well. Though it develops no fragility fractures, mechanical testing is a good surrogate. Estrogen-deplete nonhuman primates are the large animal of choice when studies of Haversian remodeling are required.

Data about bone behavior in estrogen-deplete mice are now convincing as to similarity to post-menopausal women. Knockout and transgenic mice with interesting skeletal phenotypes will necessitate full characterization of the adult mouse skeleton in the next few years. Female mice are likely to have skeletal growth and maturation phases that can make them useful for peak bone mass experiments. Mice are likely to be uniquely useful in revealing genes that control peak bone mass, showing skeletal behavior after specific gene alterations, and providing animals with “designer bone disease.”

IV. DISUSE OSTEOPENIA

A. IN GENERAL

The prevalence of human disease associated with permanent or transient skeletal disuse is rising as more persons survive partially debilitating events like stroke, myocardial infarction, and central nervous system trauma. While there are reasonable animal models of marked declines in bone use, there are no models of the imperceptible decline in mechanical usage that impacts the aging skeleton. Experimental and cohort data from humans with disuse osteopenia is first reviewed to place the data from animal models in proper perspective.

The principal conditions of skeletal disuse are paraplegia or quadriplegia after spinal cord injury, spaceflight, enforced bedrest, or stroke with varying degrees of paralysis. Information is limited by the availability of participants for studies and ethical/operational considerations in experimental design. Disuse osteopenia was first described at the same time as post-menopausal osteoporosis.¹⁷¹ Long-term disuse is characterized by localized bone mass decreases of ~30%, implied bone formation rate decreases of ~80%, and the replacement of red by fatty marrow.^{172–173} Bone mass several years after spinal cord injury is reduced 40–60%.¹⁷⁴ In limbs immobilized one year after stroke, bone mineral density is 4–8% lower than in the opposite limb.^{175–176} Transient disuse osteopenia has been documented following fracture,¹⁷⁷ but its etiology is intertwined with the RAP.¹⁷⁸

The main disuse experiment in humans is bedrest in healthy fourth decade males.^{179–187} All other data are from cohort studies.^{172–173,175,188–191} Since few opportunities occur to observe the development of steady state disuse osteopenia in humans, its pathophysiology is poorly characterized. Four month bedrest experiments in healthy men imply transient elevation of resorption.¹⁷⁹ However, this elevation and a likely decline in formation¹⁸⁰ causes only a 1–10% loss of bone,¹⁸¹ that is completely regained within seven months. Thirty to fifty percent bone loss is reported following bedrest for treatment of back pain.^{189–190} Urinary calcium rises transiently in humans

TABLE 2
Summary of Various Immobilization Methods in Rats

Method	Advantages	Disadvantages
Sciatic Neurectomy and Others	Profound immobilization; straightforward; widely-used; long-term studies possible	Irreversible; proper sham surgery difficult (RAP); involvement of nerve changes; no monitoring of food consumption; foot chewing; mostly in growing rats; sham-op control and study of both limbs not done
Tail Suspension	Reversibility; non-surgical; consistency of findings; spaceflight relevance; ease of drug intervention	Unique equipment and confinement; only growing animals possible; short term, labor intensive; food intake never monitored; muscle motion possible
Hindlimb Taping	Reversibility; non-surgical; consistency of findings; simple equipment; suitable for growing and adult rats ease of drug intervention; long-term studies possible	High maintenance; leg motion possible; food intake never monitored
External Fixation	Reversibility; non-surgical; operator specific; suitable for growing and adult rats; ease of drug intervention; long-term studies possible	High maintenance; leg motion possible; food intake never monitored
Tenotomy	Partially reversible; straightforward; suitable for growing and adult rats; ease of drug intervention	Sham-surgery; only short-term studies possible w/o intervention to prevent healing
Spaceflight	Reversibility; non-surgical; true weightlessness	Muscle motion permitted; inconvenient; food intake effect not reported

during spaceflight.¹⁹¹ The human data suggest that a few months' immobilization causes mild, reversible bone loss that is unlike that seen with severe immobilization following permanent injury. Thus, the descriptive pathophysiology of the development of acute disuse osteopenia in humans is incomplete, making the development of *in vivo* animal models somewhat difficult.

Though a few disuse osteopenia studies have been done in dogs, goats,¹⁹² turkeys, and monkeys,¹⁹³ the principal animal is the rat. Permanent and transient methods exist. Cross model findings are concordant, but each has problems (Table 2). Many have surgical components that cause a RAP.¹⁷⁸ The transient ones require continuous attention to maintain substantial, though incomplete immobilization, creating less severe bone loss that may be relevant to milder disuse. Permanent ones give profound immobilization and are low maintenance, but preclude studying recovery, being most relevant to disuse osteopenia associated with CNS injury.

B. ANIMALS

Reversible forelimb immobilization in dogs by casting has been reported.^{194–197} Progressive cortical and cancellous osteopenia in the immobilized limb of ~20–25% develops during 10–12 weeks, followed by a slower decline to a steady state of ~30–35% by 32 weeks. Young dogs develop more rapid and severe osteopenia, but experience more complete recovery than old dogs. Full recovery takes about 1.5–2 times as long as the immobilization period in animals remobilized after 16 weeks or less.¹⁹⁸ Immobilization longer than 12 weeks prevents complete recovery in older dogs. Others using similar methods in adult dogs concur.¹⁹⁹ Others report 30–40% osteopenia in growing dogs after four weeks immobilization that is partially prevented by bisphosphonates, tamoxifen, or NSAIDs.^{200–201} There is thus good consensus that the immobilized dog forelimb responds to reversible immobilization with relative cancellous and cortical osteopenia. It is the main model for studying disuse effects on Haversian remodeling.

Permanent, surgical immobilization of one forelimb has been done in turkeys. Cortical osteopenia of ~12% develops during 4–6 weeks, and is preventable by four loading cycles per day.^{202–203} This model has not been adequately controlled by sham-operation. It also requires unique skills and equipment. Its results have not been validated in multiple independent laboratories.

Permanent and temporary immobilization models in rats are ubiquitous. Permanent models include sciatic or caudal neurectomy, hemicordotomy, and amputation.²⁰⁴ Temporary models include hindlimb taping; external fixation, including casting or tail encasement; tenotomy; hindlimb suspension; and spaceflight.

C. COMMON PROCEDURES

1. Neurectomy

Neurectomy is the most frequently-used model for inducing disuse osteopenia. A 5 mm section of the sciatic nerve is resected, causing permanent distal paralysis of the operated limb. Immobilization times from 1–8 weeks have been studied.^{205–214} It is suitable for both growing and adult rats, but is most frequently used in growing rats. Cancellous bone loss of 10–15% occurs after 10 days²⁰⁷ that stabilizes at 40–75%^{205–206} by four weeks. Loss is more rapid and prominent in cancellous than cortical bone.^{205,209} The most prominent finding is decreased periosteal bone formation.²⁰⁵ Bone formation is decreased while bone resorption is increased in cancellous bone.^{205,206} While bone formation is universally reduced, bone resorption endpoints occasionally are found unchanged.²⁰⁷ Sciatic neurectomy in older rats may cause both limbs to lose bone with more marked loss in the operated limb.²⁰⁸ This systemic bone loss may also be an effect of reduced food intake or lack of movement associated with hindquarters injury. Various interventions prevent the bone loss induced by sciatic neurectomy.^{205–206,208} At three weeks, caudal neurectomy causes depressed bone formation, but only minor bone loss.²¹³ Hemicordotomy causes more rapid and severe loss of bone than does sciatic neurectomy.²¹⁰

2. External Fixation

Several reversible methods are available. Recovery from steady state immobilization is an excellent method to study cellular events during natural bone formation stimulation. These immobilization methods tend to be laboratory specific. The hindlimb taping model is most frequently applied.^{215–222} One hindlimb is secured against the ventral body wall by adhesive tape. The tape is checked daily and replaced weekly as the immobilized limb is stretched. Studies of up to 26 weeks exist. Older rats are most frequently used, but it is suitable for growing rats. Cancellous bone loss of 10–12% is detected by two weeks that progresses to 20–25% at six weeks,²²⁰ 45% by 10 weeks, and stabilizes at ~60% by 18 weeks.²¹⁶ For bone mineral density, loss can be detected at 10 weeks (12%) that stabilizes at ~18% by 18 weeks.²¹⁶ These changes are accompanied by a transient 50% rise in cancellous bone resorption during the bone mass decline, with an accompanying chronic 20% depression in bone formation.²¹⁶ Remobilization studies work well.^{219,222}

Another reversible method applies padded tape to one limb.^{223–225} Loss of 5–12% in BMD occurs by three weeks. Another method is by plaster cast.²²⁶ Yet another reversible method is with filament tape,²²⁷ that induces an 8–25% decline within four days in four-week-old rats, with a 40–50% decline in bone formation rate.²²⁷ Tail encasement for 10 days causes 6% reduction in caudal vertebra BMD.²²⁸ Another reversible method is suturing the knee in a partially flexed position, resulting in 10–15% loss of BMD in three month old rats within one week.²²⁹

3. Tenotomy

Tenotomy is a partially reversible surgical method of immobilization.^{230–232} Reversal occurs as healing takes place and the rat adapts. After two weeks in four-week-old rats, 15% loss in cancellous

bone volume occurs, with elevation in resorption surfaces and decline in formation surfaces.²³¹ Another study suggests 35% loss after three weeks, with a rise in resorption surfaces and no change in formation.²³² Comparative work suggests that tenotomy and sciatic neurectomy are similar.

4. Hindlimb Suspension

This model matches spaceflight duration and conditions.^{233–238} Thus it is generally applied with rats aged 4–6 wks and a duration of 4–14 days. Support of the body weight by the tail with heavier rats is troublesome. Special cages are required. The rats are suspended by the tail from a rotating arm in the cage roof that permits the rat to walk only on its forelimbs at 30° head down tilt to match spaceflight fluid shifts. Hindlimbs of suspended rats have 20–30% lower bone density and cancellous bone volume than controls after 4–14 days. Bone mass in these growing rats recovers quickly upon remobilization.^{236–237}

5. Spaceflight

Growing rats flown in space for 14 days lose 50–60% cancellous bone volume in regions accustomed to loading.²³⁹ This loss is primarily accompanied by decreased formation.²⁴⁰ Other shorter studies suggest that bone loss accompanied by decreased formation and occasional increased resorption also occurs.^{239–244} Bone mass in these growing rats recovers in 1.5–2 times the duration of spaceflight.²⁴³ Recent data suggest that spaceflight bone loss occurs in singly-housed, but not in multiply housed rats.

V. GLUCOCORTICOID OSTEOPENIA

Though human glucocorticoid (GC) osteoporosis has been recognized as both an endocrinologic and iatrogenic entity for over half a century,^{246,247} its pathogenesis remains controversial.^{248–252} It is marked by vertebral crush fractures, osteopenia with normal numbers of thinned trabeculae, and low bone formation rate.^{129,253–255} Secondary hyperparathyroidism linked to reduced intestinal calcium absorption is also found, but the major long-term lesion is low bone formation.

A. SMALL ANIMALS

Most mouse and rat experiments on GC skeletal effects have used growing animals.^{256–266} They yield the confusing result of increased metaphyseal cancellous bone mass or density with thinned epiphyseal growth cartilages, decreased bone elongation rate, and decreased rates of bone formation and resorption. The paradoxical finding of increased cancellous bone mass is due to the GC influence on the endochondral ossification process with decreased rate of disappearance of mineralized metaphyseal tissue, coupled with decreased bone elongation, leaving more mineralized tissue in a smaller bone. Low cortical bone mass and strength is also found in treated groups.^{260–262} This is due to general GC inhibition of bone formation that decreases the rapid periosteal formation in cortical bone of growing animals. While decreased bone formation is a pertinent finding, its cause is not the same as in adult human GC osteopenia. The cortical osteopenia and low bone strength of GC treated growing rats is due to reduced periosteal expansion, not the same process producing GC osteopenia in adult humans.

As in all osteoporosis research, growing animals are poor models. For GC-induced osteopenia, growing animal data has led to the erroneous conclusion that all mouse and rat experiments are inappropriate. In fact, experiments in older GC-treated rats show either a trend to, or a significant decrease in metaphyseal bone mass.^{267–274} The usual dose and treatment time is 0.5–2mg/kg/d prednisolone or equivalent for 6–12 weeks. Decreased formation rate is a universal finding. Bone density by Archimedes principle or defatted bone weight is ~6% lower^{269–271} and lumbar spine bone mineral density declines about 10% after eight weeks.²⁷⁴

Skeletal studies in rats of any age show body weight declines with GC administration. A few suggest that the food intake reduction associated with the weight decline plays some role in creating osteopenia that might be mistakenly attributed to GC actions.^{264,272} Pair-feeding of non-GC-treated rats to match the food intake of GC-treated rats is thus needed to separate the effects of GCs from those of hypophagia.

B. LARGE ANIMALS

As in post-menopausal osteoporosis studies, the rat cannot disclose all details of the GC osteopenia disease process because of its low levels of Haversian remodeling. Existing data suggest that glucocorticoids affect Haversian remodeling. Large animal studies are thus necessary to disclose the whole picture of GC skeletal effects.^{122,128,275–277} There is a phase of accelerated remodeling targeted to the endocortical one-third of the cortical bone of long bones and rib. Since a primary effect of GC treatment is suppression of bone formation, the formation phase that customarily follows the resorption phase of remodeling, proceeds extremely slowly if at all, leaving large holes, accounting for considerable bone loss.^{122,128} Other studies in rabbits suggest that bones are weaker and bone density is lower after GC treatment.^{277,278} Skeletal findings in GC-treated dogs are similar, showing a 13% loss of lumbar spine BMD over 12 months.^{128,275} Studies of glucocorticoids in sheep suggest that bone formation is suppressed significantly, but that changes in remodeling due to the photoperiod of sheep are marked.^{151,278–279}

C. SUMMARY

Past experiments with growing rats have been misleading. Older rats are likely to be an appropriate model of human GC osteopenia, but experiments that document the influence of decreased food intake during GC treatment are needed. Large animal studies have proven that Haversian remodeling is influenced by GC treatment, causing bone loss in the endocortical one-third of cortical bone. Just as for post-menopausal osteoporosis, a combination of small and large animal studies is necessary to understand its overall behavior.

VI. OSTEOPENIA ASSOCIATED WITH INFLAMMATION

A. IN GENERAL

Rheumatoid arthritis, the commonest inflammatory arthritis, is associated with loss of bone mass, alterations in bone structure, and clinically important loss of strength of the skeleton. Considerable morbidity experienced by rheumatoid arthritis (RA) patients results from the increased fracture risk associated with this disease.^{280–282} The patients are also at risk of articular surface collapse, weaker fixation of orthopaedic implants and prostheses, and periprosthetic fractures.^{283–285} Other chronic inflammatory conditions, including non-arthritic conditions, may be associated with osteoporosis and increased fracture risk.

B. ANIMAL MODELS

Arthritis and non-articular inflammation can be induced in laboratory animals to create osteopenia. These models are relevant to the morphologic and biomechanical study of bone loss, including bone remodeling, bone cellular activity, microstructure, mineralization and various aspects of bone strength. Further, the study of fixation of implants to abnormal bone requires suitable models, as does the evaluation of interventions designed to prevent or reverse bone loss and defects. Because bone quality is important in the surgical management of musculoskeletal disease, the problem of the osteopenic skeleton in RA and other inflammatory conditions that frequently require orthopaedic reconstruction is an appropriate subject for orthopaedic research.

Minne et al.²⁸⁶ described osteopenia in the rat that develops after subcutaneous injection of nonspecific irritants such as talcum (magnesium silicate) and cotton wool (cellulose), that initiate a chronic inflammation. Eight injections of 400 mg of magnesium silicate suspended in 0.5 ml saline at different subcutaneous sites on the back of female rats of the Chbb Thom strain created systemic inflammatory responses including loss of body weight, increase in spleen weight, and an increase of cells of myelopoietic origin in bone marrow. A transient decrease in circulating neutrophils, lymphocytes and monocytes was followed by an increase in peripheral blood leukocytes for the duration of the inflammation. In this model of inflammation-induced osteopenia not characterized by arthritis, there is a transient generalized depression of bone formation, measurable as early as three days after injection, due to a decrease in osteoblast function; bone resorption is not increased.^{287,288} Vitamin D²⁸⁹ and insulin,²⁹⁰ but not salmon calcitonin²⁹¹ appear osteoprotective. The model has been applied to studies of diabetes-associated osteoporosis.²⁹²

Extensive investigation into the osteopenia of inflammatory arthritis has been carried out using intra-articular injection of a sterile 1% solution of carrageenan, an extract from species of seaweed. This model was first described by Gardner²⁹³ and well characterized by others^{294–297} who applied it in studies of articular cartilage damage in inflammatory arthritis. Subsequently, the model was found suitable for studies of osteoporosis because of the consistent occurrence of erosive, rapid juxta-articular remodeling and diaphyseal bone loss, resembling the changes of RA.^{298–300} The model is described in detail in Chapter 19. Carrageenan-induced arthritis is described in mature³⁰⁰ and immature³⁰¹ dogs and rabbits.^{298–299}

C. FRACTURE RISK

A series of experiments performed in the carrageenan-injection model has elucidated mechanisms of fracture risk that may have relevance to clinical problems due to osteoporosis in RA patients. In cancellous bone, increased remodeling with bone formation increased by a factor of four, and resorption calculated to be increased by a greater ratio, results in net loss of ~20% of cancellous bone volume in 49 days.²⁹⁸ Cortical thinning was observed as well as evidence of increased remodeling in the metaphysis and diaphysis of the femur, proximal to the involved tibiofemoral joint.²⁹⁹ Since the bone was composed of a higher proportion of newly-formed material, bone mineralization was diminished compared to normal specimens.³⁰² A comparative study of bone mass and remodeling in immobilization and arthritis suggested that, in the carrageenan model, the abnormality resulted not from immobilization, but from inflammatory effects.³⁰³ The rapid remodeling osteopenia is principally observed in the bones of the ipsilateral limb,³⁰⁵ but there is inconclusive evidence that monoarticular inflammation may cause increased bone remodeling elsewhere in the skeleton.³⁰³

A further series of experiments focused on the mechanism of increased fracture risk in cortical bone. Inflammatory arthritis-induced loss of strength was demonstrated in rabbit femoral diaphyses from the limb affected by experimental tibiofemoral arthritis, showed a loss of ultimate strength of ~39%.³⁰⁴ Kang et al. documented osteopenia and decreased mechanical strength of the entire ipsilateral femur and tibia.³⁰⁵ Strips of femoral cortex from the carrageenan injection arthritis model, tested within the elastic range, demonstrated no significant alteration in the elastic and flexural modulus.³⁰⁶ Large defects in the femoral cortex were described³⁰⁷ and defined as disordered, giant resorption sites crossing osteonal borders and up to 1.2 mm in cross-sectional diameter.³⁰⁸ Cooke and Takashima identified an analogous porous lesion in the femoral neck of RA patients who underwent total hip replacement. Enlarged Haversian systems contained osteoclasts and vascular changes with tall endothelial cells.³⁰⁹ A simplified femoral shaft mathematical model predicted the loss of strength in the femoral cortex that would result from defects of the size observed in the carrageenan model. The calculated result, ~29% was reasonably concordant with the observed loss of strength (~39%).^{304,307}

These data suggest that cortical porosity resulting from focal bone resorption could be a major cause of the loss of strength of long bones in inflammatory arthritis. Since a large increase in osteoclast numbers was observed in the arthritis model,³¹⁰ a pharmacologic approach to resorption suppression was proposed as a protection against long bone fracture. Pamidronate completely prevented loss of strength associated with carrageenan arthritis.³⁰⁴ Zoledronate also prevented the formation of the giant osteonal resorption areas, supporting the hypothesis that cortical porosity due to focal osteoclastic resorptions is an important cause of increased risk of long bone fracture in inflammatory arthritis.³¹¹ These experiments also provide a basis for trials of anti-resorptives in the management of the osteoporosis of inflammatory arthritis.

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Part IV

Animal Models of Articular Cartilage and Joint Conditions



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16 Animal Models of Articular Cartilage Defect

Yuehuei H. An and Richard J. Friedman

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I. INTRODUCTION

The regeneration potential of damaged articular cartilage is extremely limited. Although many methods have been investigated, none of them have given satisfactory long-term clinical results. Over the last few decades, artificial joint replacement has developed very rapidly and many arthritic conditions have been successfully treated. However, total joint arthroplasty does not last a lifetime and, therefore, is contraindicated for most children and young active adults. A need still exists for a method that biologically regenerates full thickness defects in articular cartilage defects. With the development of tissue engineering technique, there seems to be a bright future for biological repair of cartilage injuries. Without adequate *in vitro* alternatives so far, animal models of cartilage defect are still playing a very important role at this frontier.

TABLE 1
The Total Thickness of Articular Cartilage of Femoral Condyles of Common Subjects

Species	Age	Body weight (kg)	Cartilage thickness (mm)	First author, year ^{Ref.}
Human	Adult	—	2.2–2.4	Martino 1993 ³
Cow	1 year	273	3.17	Simon 1970 ⁴
Sheep	2–5 years	68	1.68	Simon 1970 ⁴
Dog	2–3 years	25	1.3	Simon 1970 ⁴
Rabbit	12–14 months	4.5±0.25	0.25–0.75	Athanasiou 1995, ⁵ An 1998 ⁶
Rat	12 months	300 (gm)	170 µm	Simon 1970 ⁴
Mouse	12 months	22 (gm)	30 µm	Simon 1970 ⁴

II. NORMAL ARTICULAR CARTILAGE AND REPAIR PROCESS

A. NORMAL ARTICULAR CARTILAGE

Morphologically, articular cartilage is composed of four layers.¹ (1) The superficial layer, about 10% of the cartilage thickness, is composed of mainly thin collagen fibers oriented along the outer surface of the joint. The lower part of the layer contains flattened chondrocytes aligned parallel to the surface. The load-bearing ability of the cartilage depends largely upon the integrity of this layer. (2) The intermediate layer is composed of vertically oriented collagen fibers and small spherical chondrocytes. The columnar orientation is less marked than in the deep layer. (3) The third or deep layer composed of large collagen fibers and larger chondrocytes. The collagen fibers are oriented mainly perpendicularly to the joint surface. The large chondrocytes form chondrons, which are composed of several chondrocytes surrounded by matrix and an outer layer of collagen/proteoglycan capsule.² The chondrons lie in columns oriented in vertical direction. (4) The fourth or deepest layer, about 10% of the cartilage thickness, is the calcified cartilage, which joins together the cartilage and subchondral bone and provides growth and remodeling of underlying bone tissue. Collagen fibers are arranged in vertical bundles with bridging fibrils in between. There is a basophilic line called “tidemark,” aggregates of mineral associated with matrix vesicles, separating the uncalcified cartilage from the calcified layer. The total thickness of articular cartilage of femoral condyles of human and common animal subjects are listed in Table 1.

Biochemically, articular cartilage is composed of hydrated extracellular matrix (ECM) (collagen, proteoglycans [PGs], and noncollagenous proteins) in which chondrocytes are embedded.^{7,8} Chondrocytes occupy only less than 1% to 10% of the tissue volume. A particular feature of these chondrocytes is that they lack contact between cells; thus, communication between cells are through the ECM. There are no blood and lymphatic vessels for the delivery of nutrients to and the removal of wastes from the cells, so these functions are performed via diffusion through the ECM. There are no nerve fibers in cartilage. Chondrocytes live in an anoxic environment and seem to carry out their metabolism mostly through anaerobic pathways. The collagen, 90% type II collagen and 10% type IX, X, XI, and VI collagen, represents more than 50% of the organic dry weight or 10–20% of the wet weight. The network of type II collagen fibrils provides the tensile strength of cartilage and is essential for maintaining tissue volume and shape. PGs, including aggrecan, biglycan, and decorin, are a diverse group of heterogeneous macromolecules. Each type of PG is composed of a protein core and a number of glycosaminoglycan (GAG) chains. The most important GAGs are chondroitin sulfate, keratan sulfate, dermatan sulfate, and heparan sulfate. As many as 100 aggrecan and link proteins can bind to a strand of hyaluronan to form a large aggregate. PGs function as a space filler, provide swelling pressure by attracting water, and assist the mechanical properties of the collagen network. Articular cartilage also contains a number of extracellular noncollagenous matrix

proteins, such as chondrocalcin, anchorin, fibronectin, or thrombospondin. These proteins may be involved in the process of cartilage calcification, interaction between chondrocytes, tissue repair and remodeling.

Biomechanically, cartilage is considered to be a multilayered, heterogeneous, anisotropic, physically non-linear, viscoelastic, 2-phase porous structure. The average equilibrium compression modulus for the lateral condyle and femoral groove cartilages of normal human, canine, monkey, and rabbit and bovine meniscus are 0.41–0.89 MPa.^{9,10} The average equilibrium tensile modulus by tensile test of human, bovine and canine cartilage is 1.0–15 MPa.^{9,11} The aggregate modulus by indentation test is 0.81–1.82 MPa for cartilage of human femoral head¹² and 0.59±0.18 MPa for rabbit femoral condyles.⁵ The average equilibrium shear modulus by torsional test of human patellar cartilage is 0.23 MPa.¹³ The hydraulic permeability of cartilage is 0.63±0.28 × 10⁻¹⁵ m⁴/N.sec (rabbit femoral condyle) by confined compression test¹⁰ and 0.71–1.10 m⁴/N.sec (human femoral head).¹²

B. REPAIR PROCESS OF CARTILAGE DEFECT

Unlike the repair process of other tissues, articular cartilage does not heal satisfactorily by itself although some studies have demonstrated close to complete repair with hyaline cartilage in immature animals or in small defect.^{14,15} DePalma et al.¹⁶ reported that partial-thickness defects show no significant repair up to more than one year, but that full-thickness wounds were completely filled with immature cartilage in four months. Campbell found that injuries to hyaline cartilage do not heal with normal hyaline cartilage, but mainly with fibrous tissues or fibrocartilage.¹⁷

Silver and Glasgold⁸ pointed out that three factors are important in determining whether cartilage repair occurs: (1) the depth of the defect, (2) the maturity of the cartilage (better results in young subjects), and (3) the position of the defect on the surface. It is obvious that the size of the defect is also a determining factor of cartilage repair.^{15,18} Significant better repair of full-thickness defects than partial-thickness defects indicated that the repair process appeared to be mediated by the proliferation and differentiation of mesenchymal cells of the marrow, and not by chondrocytes of the defect wall.^{16,19} The repair of full thickness defects follows the following sequence: fibrin; granulation tissue; connective tissue; cartilage cells in connective tissue; fibrocartilage; and hyaline cartilage.^{16,20} The ultrastructural changes of cells and matrix in the defect during the repair process have been reported by Ghadially et al.²¹ In conclusion, cartilage only has an incomplete capacity for self-repair.

III. ANIMAL MODELS FOR CARTILAGE REPAIR

A. HETEROTOPIC MODELS OF CHONDROGENESIS

One of the major heterotopic models for *in vivo* chondrogenesis to test potential substances includes the subcutaneous, intramuscular, and intraperitoneal model (Table 2). After promising *in vitro* studies such as cell culture or cell seeding, subcutaneous implantation is often the initial step of *in vivo* studies. The animals used for heterotopic models include nude mice, syngenic mice, rats, and rabbits. Substances or constructs having potential effect of chondrogenesis were implanted or injected at the above-mentioned sites. After 2–4 months the implants were explanted and studied histologically to identify new cartilage tissues. Although Green³⁵ found similar cartilage formation at both of the intramuscular and subcutaneous model, the effects of implantation sites and local environment are still not clear. Older rabbits (3.2–4.0 kg) were grafted with chondrocytes from younger rabbits (2.2–3.2 kg) into the anterior tibial compartment.³⁵ The result showed that freshly isolated articular chondrocytes reformed cartilage tissue in 10 days.

Another heterotopic defect model of chondrogenesis is a diffusion chamber implanted intramuscularly in rat or nude mice (Table 1). The diffusion chamber is made from a plastic ring (2 mm thick, 9 mm diam.) bounded by two microporous cellulose acetate and nitrate membranes of

TABLE 2
Selected Heterotopic Models of Chondrogenesis

Animal	Procedure	Material tested	First author, year ^{Ref.}
Nude mice	SC*	Human periosteal cells-porous ceramic	Nakahara 1991 ²²
		Chondrocytes of rabbits and dogs	Lipman 1993 ²³
		Chondrocyte-PGA/PLA construct	Vacanti 1991 ²⁴
		Chondrocyte-PGA/PLA construct	Freed 1993 ²⁵
		Chondrocyte-PGA/PLA construct	Puelacher 1994 ²⁶
		Chondrocyte-calcium alginate construct	Paige 1996 ²⁷
		Chondrocyte-collagen composite	Fujisato 1996 ²⁸
Syngenic mice	IP†	Chicken periosteal cells in diffusion chamber	Nakahara 1990 ²⁹
	IP	Human periosteal cells in diffusion chamber	Nakahara 1991 ²²
	IP	Rodent Achilles tendons in diffusion chamber	Rooney 1993 ³⁰
Rats	IM‡	Syngenic rat chondrocytes	Moskalewski 1993 ³¹
	IM	Rabbit BMP in diffusion chamber	Ono 1994 ³²
	SC	BMP carriers	Kuboki 1995 ³³
Rabbits	SC	Collagen sponge plus perichondrium	Matsuda 1995 ³⁴
	IM	Chondrocytes from younger rabbits	Green 1977 ³⁵

* SC = Subcutaneous tissue

† IP = Intraperitoneally

‡ IM = Intramuscularly

100 μ m thickness and 0.45 μ m pore size and a chamber volume about 130 μ l (Millipore Corporation, MA).^{22,29,30,32} A diffusion chamber containing rabbit bone morphogenetic protein (BMP) was implanted in the abdominal muscle of the rat. Outside of the chamber, cartilage differentiated 1–2 weeks after implantation, and bone replaced the cartilage after 3–4 weeks.³²

B. CARTILAGE DEFECT MODELS

Selected cartilage defect models from the literature are listed in Table 3. There are mainly two types of defects, focal full thickness defect and partial-thickness defect. The commonly used animals are rabbits and dogs which are the first choices for the studies on cartilage repair.

IV. AUTHORS' PREFERRED ANIMAL MODELS

A. HETEROTOPIC MODELS (NUDE MICE MODEL)

The nude mice model for testing *in vivo* chondrogenesis of potential substance or construct is the most popular heterotopic model for chondrogenesis.^{27,28} Athymic nude mice with an average age of 5 \pm 1 weeks could be selected. A total of 2–4 sample disks (9 mm diam., 2 mm thickness) can be implanted into noncommunicating subcutaneous pockets on the back of the mouse. The substance can be mixed with collagen gel or other gelly carrier and injected into SC tissue. Care needs to be taken where the material is inserted or injected, which should be in the loose SC tissue and not underneath the back muscles. Histological evaluation is efficient to examine the existence of new cartilage tissues.

B. RABBIT DISTAL FEMORAL JOINT DEFECT

The basic steps in the testing of the cartilage repairing effect of an implant or construct include the manufacturing of the implant, the creation of a cartilage defect, the placement and securing of

TABLE 3
Selected Animal Cartilage Defect Models

Animal	Bone	Types of defect	First author, year ^{Ref.}
Rabbit	Distal femur	Round drill hole (s)	Ghadiaily 1971, ²¹ Green 1977, ³⁵ Speer 1979, ³⁶ Furukawa 1980, ³⁷ Salter 1980, ³⁸ Shimizu 1987, ³⁹ Heatley 1985, ⁴⁰ Aston 1986, ⁴¹ O'Driscoll 1986, ⁴² Amiel 1988, ⁴³ Wakitani 1989, ⁴⁴ Lipiello 1990, ⁴⁵ Dahlberg 1991, ⁴⁶ von Schroeder 1991, ⁴⁷ Hogervorst 1992, ⁴⁸ Shapiro 1993, ¹⁹ Klompmaker 1992, ⁴⁹ Messner 1993, ⁵⁰ Messner 1994, ⁵¹ Freed 1994, ⁵² Kreder 1994, ⁵³ Chu 1995, ⁵⁴ Kandel 1995, ⁵⁵ Sumen 1995, ⁵⁶ Specchia 1996, ⁵⁷ Wei 1997, ⁵⁸ Frankel 1997, ⁵⁹ Sellers 1997 ⁶⁰
			Kawabe 1991, ⁶¹ Wakitani 1994, ⁶² Brittberg 1997, ⁶³ Chu 1997 ⁶⁴
			Menche 1996 ⁶⁵
			Mochizuki 1993 ⁶⁶
			Hunziker 1996 ⁶⁷
			Bentley 1978, ⁶⁸ Kon 1981 ⁶⁹
			Upton 1981, ⁷⁰ Grande 1989, ⁷¹ Muckle 1989, ⁷² Brittberg 1996, ⁷³ Specchia 1996 ⁵⁷
			Chesterman 1968 ⁷⁴
			Calandruccio 1962, ¹⁴ Klompmaker 1992, ⁴⁹ Hale 1993, ⁷⁵ Shortkroff 1996, ⁷⁶ Breinan 1997 ⁷⁷
			Campbell 1963 ⁷⁸
Dog	Proximal tibia	Rectangular or oval	Menche 1996 ⁶⁵
	Patella	Large full-thickness	Mochizuki 1993 ⁶⁶
		Scalpel, superficial	Hunziker 1996 ⁶⁷
		Small partial-thickness	Bentley 1978, ⁶⁸ Kon 1981 ⁶⁹
		Round hole	Upton 1981, ⁷⁰ Grande 1989, ⁷¹ Muckle 1989, ⁷² Brittberg 1996, ⁷³ Specchia 1996 ⁵⁷
Dog	Humerus	Wedged osteotomy	Chesterman 1968 ⁷⁴
	Distal femur	Round or oval hole	Calandruccio 1962, ¹⁴ Klompmaker 1992, ⁴⁹ Hale 1993, ⁷⁵ Shortkroff 1996, ⁷⁶ Breinan 1997 ⁷⁷
	Distal femur	Condyle osteotomy	Campbell 1963 ⁷⁸
	Distal femur	Superficial	Calandruccio 1962 ¹⁴
	Patella	Chondral excision	Engkvist 1979 ⁷⁹
Rat	Distal radius	Osteotomy	Campbell 1963, ⁷⁸ Steveson 1989 ⁸⁰
	Distal femur	1.0–1.5 mm hole	Noguchi 1994, ⁸¹ Grundnes 1995, ⁸² Chang 1996 ⁸³
		1.2 × 5 mm defect	Göransson 1995 ⁸⁴
Goat	Tibial plateau	Wedged defect	Jackson 1993 ⁸⁵
	Fem. condyle, patella	3.0 mm holes	Shahgaldi 1991 ⁸⁶
	Femoral condyle	4.0 mm round hole	Butnariu-Ephrat 1996 ⁸⁷
Horse	Distal femur	Circular hole	Hendrickson 1994, ⁸⁸ Sams 1995 ⁸⁹
	Radial carpal bone	Circular hole	Vachon 1992, ⁹⁰ Howard 1994 ⁹¹
		Rectangular	Todhunter 1993 ⁹²
	3rd carpal bone	Partial-thickness defect	Shamis 1989 ⁹³
Primate	Mandibular condyle	Round hole	Robinson 1993 ⁹⁴
	Femoral condyle	1.5 mm holes	Girdler 1993 ⁹⁵
Chick	Tibiotarsal joint	3–4 mm round hole	Itay 1987, ⁹⁶ Robinson 1990 ⁹⁷
Sheep	Femoral condyle	5 × 10 mm defect	Homminga 1991 ⁹⁸

the implant, specimen harvesting, and evaluation. The authors prefer the rabbit knee model with a defect on the distal femoral joint surface since (1) it has been widely used and well studied, (2) has a good size for easier surgical procedures and specimen handling, (3) is consistently reproducible, and (4) is relatively economical. By comparison, if there are no specific reasons, rats are perhaps too small for manipulation and specimen handling and dogs are more difficult to be justified ethically and economically.

Theoretically, there would be four major cartilage defect models including localized or extensive full-thickness defect with a depth beyond the subchondral bone plate, and localized or extensive partial-thickness defect within the cartilage layer. The localized models are suitable for investigating

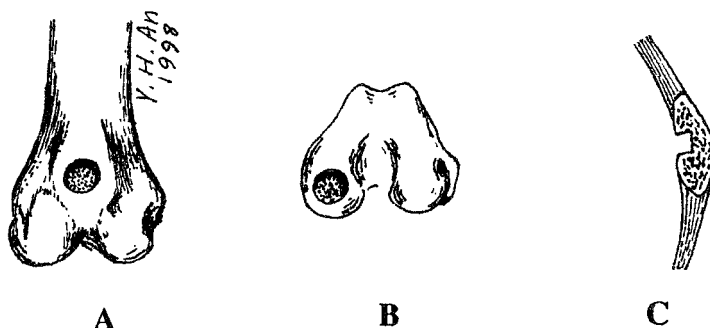


FIGURE 1. Illustration of commonly used articular defects in the rabbit: (A) intercondylar groove (partial weight bearing), (B) femoral condyle (weight bearing), and (C) patellar articular defect.

the effect of an implant or construct and the extensive ones are often used for examining other methods such as abrasion burr arthroplasty, enzymatic treatment,⁶⁶ or joint surface drilling. The rabbit knee models can be classified into weight-bearing and “partial-weight bearing” models. The former include the models with the defect created on the distal femoral joint surface, especially those located inferioposteriorly (rabbit knees stay in a flexed position most of the time). The latter are those with the defects created in the intercondylar groove.

Typically, the rabbit is put in a supine position and the knee joint is opened through a lateral parapatellar incision. The patella is dislocated medially to expose the articular cartilage of the patellar groove and the femoral condyles. A 3.5 mm diameter and 1.5–3.0 mm deep defect is created using a drill in either the intercondylar groove or the central portion of the lateral or medial femoral condyles (Figure 1). At least a 1.25 mm depth is needed if a full thickness defect is to be made since the total thickness of the cartilage and the subchondral bone plate is 1.0 mm in average for femoral condyle joint surfaces. It is a good idea that the deeper depth (3.5–5.0 mm) is created to facilitate the anchoring of a cylindrical implant (Figure 2A).⁵² Implants or constructs can also be secured with sutures (Figure 2B). If gel materials, such as collagen gel containing chondrocytes, are to be used, the gel can be maintained in place with a piece of periosteum sutured to the defect rim (Figure 2C).

C. THE SECOND CHOICE

If there are reasons of inappropriateness of using the rabbit model, a dog distal femur defect model may be selected. The dog knee joint is much larger than that of rabbits, allowing a larger defect. Also, because of the larger volume of cartilage it is easier to harvest enough cartilage tissue for chondrocytes isolation used in tissue engineering approach for autogenic cell seeding. In our laboratory, we experienced the difficulty of culturing chondrocytes based on a small piece of cartilage tissue taken from a 3.5 mm diam. cartilage defect in a rabbit model (at least 50% failure).

V. EVALUATION OF CARTILAGE DEFECT REPAIR

A. MACRO FINDINGS AT NECROPSY

The surface morphology of the repair tissue and adjacent cartilage should be recorded descriptively. Any limitation of joint movement, joint swelling, or degenerative changes (erosion of cartilage surface or formation of osteophytes) should be also documented. For most articles, macro photographs of the

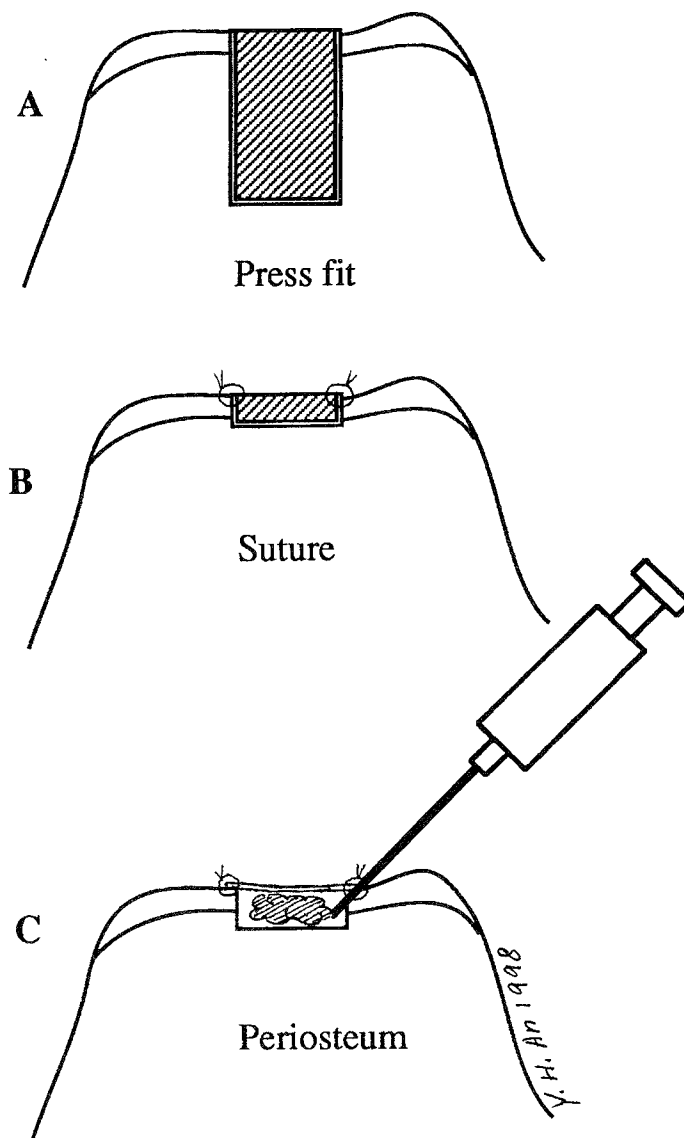


FIGURE 2. Illustration of three commonly used implant anchoring methods for repairing cartilage defects: (A) press fit, (B) suturing, and (C) periosteum (for gel materials).

specimen are basic for the results section, which give a general image of the quality of the repair. Photography of the repaired cartilage surface taken under dissecting microscope is also a useful documentation of the surface morphology. Although descriptive macro findings are important, no grading system has been reported.

B. HISTOLOGY AND HISTOMORPHOMETRY

Histology and histomorphometry may be the most powerful methods for examining the quality and quantity of the repair. Traditional histology is descriptive and semiquantitative. Two original grading systems can be found in the literature, the system described by Pineda et al.⁹⁹ and the one by O'Driscoll

TABLE 4
Modified Histological Grading System from the Original Systems in the Literature*

Category	Scores
Nature of the repair tissue	
Hyaline cartilage	4
Mostly hyaline cartilage	3
Mostly fibrocartilage	2
Mostly non-cartilage	1
Non-cartilage only	0
Matrix staining (Safranin-O)	
Normal staining	3
Moderate	2
Slight	1
None	0
Structural integrity	
Normal structure	2
Slight disruption	1
Severe disruption	0
Surface regularity	
Smooth and intact	2
Slight disruption	1
Severe disruption	0
Filling of the defect	
100%	2
>50%<100%, or >100%	1
<50%	0
Bonding to host tissue	
Bonded	2
Partially bonded	1
Not bonded	0
Degenerative changes of the repair tissue	
Normal cellularity and cell morphology	3
Mild hypocellularity and cell cluster	2
Moderate hypocellularity and cell degeneration	1
Severe hypocellularity and cell degeneration	0
Degenerative changes of the adjacent cartilage	
Normal cellularity, cell morphology, and matrix staining	3
Mild hypocellularity and cell clustering, moderate matrix staining	2
Moderate hypocellularity and cell degeneration, decreased matrix staining	1
Severe hypocellularity and cell degeneration, poor or no matrix staining	0
Maximum Possible Score (The most abnormal condition)	21

* Sources: O'Driscoll et al.,^{48,100} Pineda et al.,⁹⁹ and Freed et al.⁵²

et al.^{42,100} They have been used by other investigators with modifications.^{52,62} The two systems have common categories and each has its own advantages. A combined system may be more complete, which is proposed here for investigators who may be interested (Table 4). The mean and standard deviation are calculated for the individual categories and the total score for each of the graded specimens. The Fisher exact test, chi-square test, or Kruskal-Wallis test (a one-way non-parametric analysis of variance) could be used for analyzing the differences between the scores of different groups.^{42,101}

In recent years, computerized image analysis makes histomorphometry more efficient, especially for the calculation of the percentage of filling by the repair tissues in the defect and the fractions of different tissues in it. Normally, the parameters for quantification include the percentage of total area of the defect that become filled with repair tissue, different types of tissues (hyaline cartilage, fibrocartilage, fibrous tissue), the integration of repair tissue with adjacent cartilage and the calcified cartilage at the base of the defect.⁷⁷ Yoshioka et al.¹⁰² defined three parameters for quantifying cartilage defect repair: repair height, percent repair, and root mean square roughness. The later is a quantitative measure of the deviation of repair site surface from an idealized surface. See Chapter 6 for more information on cartilage histomorphometry.

C. MECHANICAL TESTING

A common method for mechanical testing of cartilage tissue is the confined compression test.^{64,103} A cylindrical sample is tested by being placed in a PBS bath within a confined compression apparatus with the articular surface against a stainless steel filter and the subchondral surface supported on a rigid flat disc. The mechanical property of the cartilage is described by equilibrium confined compression modulus, which is about 0.41–0.89 MPa.^{9,10}

Another important test is the indentation test which has been widely used to examine the mechanical properties of the repaired tissue.^{62,104,105} Briefly, a needle-penetration technique is used to measure the thickness of the cartilage at the area to be tested for indentation depth. The force on the needle and its displacement are measured simultaneously so the thickness of the cartilage can be documented. For the indentation test, a 1.5-mm porous indenter is used to first apply a 2 gm preload to ensure uniform contact between the indenter and the cartilage surface. Then, an additional 2 gm load is applied to record the displacement of the indenter. The mechanical parameter to be measured is the compliance value, which is calculated using the following equation: Compliance = 1/stiffness = $k \times \text{indentation depth} / \text{cartilage thickness}$ (k is an instrument constant that was the same for all tests). Normal cartilage has small values (usually single digits), implying low compliance or high stiffness, while softer or unnormal tissues have a larger number (usually two digits), indicating high compliance or low stiffness.

Elastic modulus or compliance values should be measured for the repair tissue, the adjacent cartilage, and the same areas in the contralateral knee. The means of the values of the repair tissue or the adjacent cartilage of different groups can be compared using Student's *t*-test or ANOVA test.

D. OTHER METHODS

SEM (scanning electron microscopy) is an important method for examining morphology and surface structure on joint surface and cross-sectional surfaces of cartilage. The shortcoming of SEM is that the specimen has to be dried before observation, which deforms the real spatial structure and morphology.¹⁰⁷ This problem seems to have been solved by low-temperature or cryo-SEM systems.^{107,108} TEM is the best method for examining ultrastructure and components of cartilage including matrix, chondrocytes, organelles, membranes, and large molecules (such as proteoglycan or collagen fibrils).^{109–111} See Chapter 6 for more information on TEM techniques.

The relative amounts of type I and II collagen comprising the repair tissue can be determined by gel filtration high performance liquid chromatography (HPLC).^{64,112} Total GAG content in the repair tissue is measured with a hexosamine method.¹¹³ DNA content and synthesis can be measured with a fluorometric day assay¹¹⁴ and ³H-thymidine method.¹¹⁵ See Chapter 6 for more biochemical assays for cartilage tissue.

VI. GRAFTS INVESTIGATED FOR CARTILAGE REPAIR

A. AUTOGRAFT, ALLOGRAFT, AND XENOGRAFT

Although there were promising results from animal studies,^{14,78} the use of autogenous tissue grafts to repair localized cartilage defects has been associated with the difficulty of requiring another surgical procedure and the risk of damaging the donor site. Obviously, there is a very limited amount of autologous cartilage available for grafting in humans. Also, the overall preservation of cartilage before transplantation seems to have an adverse effect on its mechanical and morphological properties, and on a long term basis has proven to be less than satisfactory.^{78,116,117}

The major problem associated with allogenic cartilage grafts is the formation of loose vascular fibrous tissue surrounding the grafts.^{41,68,74} Osteochondral allografts are easy to anchor into the recipient site, but tend to cause a severe immunologic rejection from the host.^{85,118} For some post-traumatic joint injuries or limb salvage procedures in tumor cases, allografts still play an important role.^{119,121}

Periosteal grafts,^{42,122} perichondrial grafts,^{70,79} bone callus, and cortical bone⁸⁴ have been reported as alternative methods to repair cartilage defects by introducing chondrogenic or progenitor cells to the site of repair. Cartilage components such as hyaline cartilage or fibrocartilage have been generated in the defects of experimental animals and human patients.¹²³ Long-term results, however, have not been reported.

One major advantage of the xenograft in the studies of cartilage repair is that a large amount of cartilage tissue could be obtained inexpensively from a slaughterhouse. Limited number of reports on animal models of repairing cartilage defects using xenograft materials have been found in the literature with controversial results.^{86,98}

B. BIOMATERIALS

Various synthetic materials have been investigated as replacement materials for a cartilage defect. Elastomeric materials such as silicone rubber have replaced articular cartilage in prosthetic finger joints, but have limited fatigue resistance which limits their use in weight-bearing conditions.¹²⁴ Other elastomers such as hydrophilic polyurethanes,^{49,124} polytetrafluoroethylene (PTFE), and polyester,⁵⁰ have been tested as cartilage replacements to provide increased compliance and greater mechanical compatibility. However, these materials did not display sufficient fatigue resistance for long-term use, and also induce degeneration of the bearing articular cartilage. Poly (HEMA) hydrogel,⁶⁹ PLA matrix,⁴⁷ TCP-collagen composite,⁴⁸ and carbon fiber pads⁷² have also been tried. Although there have been many attempts on the development of artificial cartilage, currently there is no acceptable synthetic material for use in this type of application, i.e., a high strain to failure elastomeric biomaterial with high fatigue resistance, low friction, low wear characteristics, and more significantly, acceptable long-term results.

C. STIMULUS FOR CHONDROGENESIS

Growth factors (GFs) play a very important role in the process of chondrogenesis. The major GFs with chondrogenic ability include TGF- β , FGF, EGF, IGF, and BMPs.^{125,126} They stimulate DNA synthesis, chondrocyte proliferation and differentiation, and matrix production.^{127,128} Several carriers or substrates have been reported to deliver GFs for *in vivo* chondrogenesis studies such as DBM,¹²⁹ and collagen.²⁸

Continuous passive motion (CPM) has been used alone or in combination with osteoperiosteal grafts and demonstrated its positive effects on the formation of chondral tissue and the boundary between graft and defect.^{38,42} Kim et al. found that full-thickness defects created by subchondral abrasion can heal by regeneration of hyaline-like cartilage and such healing is enhanced by CPM for two weeks postoperatively.¹³⁰

Also, some evidence indicates that chondrocytes and progenitor cells respond to electrical or electrical field stimulation.^{131,132} A pulsing direct current stimulation was also studied for *in vivo* chondrogenesis and the results showed an enhanced quality of repair.⁴⁵

D. CHONDROCYTES OR CHONDROGENIC CELL GRAFTING

Chondrocytes or chondrogenic cells (or mesenchymal progenitor cells) have been isolated or culture-expanded from articular cartilage^{44,71,74} growth plates,^{61,68,96} perichondrium,⁶⁴ bone marrow,⁶² or periosteum.⁶² These cells were then transplanted into articular defects, secured by a piece of periosteum,¹³³ or embedded in collagen gel. These attempts have had mixed results. However, the earlier work by Grande et al.⁷¹ in 1989 had already shed some light on the development of tissue engineering technique during the later years.

E. TISSUE ENGINEERING TECHNIQUE

Cell seeding to a substrate or scaffold for making a composite graft is not a new concept,^{34,134–136} but it has not been well developed until the early 1990s, especially in the field of bone and cartilage repair. Green was the first to use this concept for repairing articular cartilage defects.¹³ In the last five years, several groups have reported very important data on the use of cell-seeded implants for repairing cartilage defects.^{24,25,52,59,64,88,137} All these studies have demonstrated that the cell-seeded composite implants induce cartilage formation leading to the final defect repair. This technique has been referred as “tissue engineering” by Langer and Vacanti.^{138,139} It has certain advantages over the previous methods, such as autogenic characteristics if the cells are taken from the same individual, minor morbidity of the donor site, and the unique properties produced in large quantities *in vitro*. Also, there is no risk of disease transmission.

Different scaffolds for cell-seeding have been used in cartilage defect repair, including porous collagen,⁵⁹ PGA mat,²⁵ porous PLA,^{25,64} or PLA-PGA copolymer mesh.²⁴ Among them, porous collagen sponge has been used widely as a substrate or scaffold for cultured cell ingrowth to make implantable composite grafts, which is not only for repairing cartilage,^{24,59,88,137} but also for other purposes such as bone defect repair, skin grafting, or esophageal grafting.

In summary, an ideal chondral graft substance should have the following properties, which most of the current methods lack: (1) the chondroconductive ability for the ingrowth of new chondral tissue; (2) the presence of differentiated autogenic chondrogenic cells for immediate chondrogenesis; (3) the presence of pre-existing GFs for chondroinduction, which convert mesenchymal or undifferentiated cells into cartilage-forming cells; (4) no donor site morbidity and no risk of disease transmission; and (5) the ability to be produced in large quantities *in vitro* and easily fabricated into any desired shape. Using tissue engineering techniques, a composite graft made *in vitro* according to the above specifications could be an optimal substance to heal a cartilage defect *in vivo*.

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17 Animal Models of Meniscal Repair

Jan Klompmaker and René P. H. Veth

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I. INTRODUCTION

Once menisci were described as the functionless remains of leg muscle¹ and it was generally believed that they could be removed without consequences. The immediate results of meniscectomy were, and still are, very satisfactory and, therefore, removal of a meniscus, when causing symptoms, was standard practice. Fairbank² was the first to call attention to the fact that removal of a meniscus is followed by roentgenographic signs of joint degeneration. He discussed the possibility that menisci

have a significant role in weight-bearing and suggested that meniscectomy results in specific degenerative changes of the knee joint. The functions attributed to the meniscus are primarily load bearing, stress distribution and attenuation of shock waves within the knee joint, thus protecting the underlying cartilage from concentrations of stress and overloading.^{3,4,5,6,7} Also, menisci are reported to increase joint congruency and facilitate the rotation of the opposing articular surfaces of the knee joint.⁸ Providing stability is a secondary meniscal function when ligamentous injuries exist.^{5,9,10} It has been proposed, although not proven, that the menisci also play a role in the distribution of lubricating fluid between femoral and tibial articular surfaces thus facilitating the lubrication of the joint.¹¹ Meniscectomy results in abnormal high stresses on articular cartilage in the meniscectomized compartment, which, over time, may provoke degenerative changes.^{4,12} Since Fairbank's observations, it has been demonstrated many times by long-term surveys of meniscectomized patients, as well as by experimental studies using laboratory animals, that removal of a meniscus results in degeneration of articular cartilage in a high percentage of cases.^{13,14,15,16} The increasing awareness of the consequences of meniscectomy has led to a more conservative approach in the treatment of meniscal lesions. Since it has been shown that the degree of degenerative changes is directly proportional to the amount of meniscal tissue removed, and is inversely related to the amount of fibrocartilage remaining^{17,18} it is good clinical practice to preserve as much functional meniscal tissue as possible while addressing the clinical symptoms caused by tears. This can either be achieved by performing a partial meniscectomy or by repairing meniscal lesions. Total meniscectomy should be considered only in the relatively rare instances in which the extent of meniscal damage is so great that partial meniscectomy or meniscal repair is not appropriate.

Repair of meniscal lesions is the other alternative and may be the best option since all meniscal tissue can be preserved. King's experiments in 1936 set the biological limitations of meniscal healing. He showed that for meniscal lesions to heal, they must communicate with the peripheral vascular area of the meniscus.¹⁸ Later it was established that only the outer 10–25% of the human's and dog's meniscus, throughout its attachment to the joint capsule, is vascularized (Figure 1) and this explains why there is no tendency for healing when tears occur in the avascular central part of the meniscus.^{19,20} As a result only lesions limited to the vascular periphery can be repaired adequately by simple suturing. For lesions situated in the avascular central part of the meniscus no reliable methods exist, although new experimental techniques are in development. Successful repair of peripheral meniscal lesions by suturing or abrasion of parasynovial tissue has been widely applied with good results in animal experiments and in clinical trials on humans.^{21,22} For repair of lesions in the avascular part of the meniscus the basic principle is to improve the vascularity of the defect by stimulating ingrowth of vascular tissue. Creation of a radial access channel from the vascular periphery towards a lesion is not very successful due to occlusion of this conduit, causing an insignificant increase in vascularity.²³ Repair by interposition of a synovial flap between lesion and periphery does stimulate healing but repair takes place by ingrowth of fibrous repair tissue not resembling meniscal fibrocartilage.^{25,25} The peripheral rim can be resected back to a bleeding bed after which the meniscus can be reattached. However, when using this method, the cross-sectional area of the meniscus is diminished and load transmission is impaired, similar to partial meniscectomy.^{23,26} Repair with fibrocartilage, resembling normal meniscal tissue, can be obtained after application of a fibrin clot.²⁷ A combination of fibrin glue and endothelial cell growth factor appeared to provide similar results.²⁸ However, it remains to be determined whether these methods are applicable for repair of large and highly stressed lesions.

After it had been shown that an access channel connecting a lesion in the avascular part of the meniscus to the periphery can result in healing of the tear, Veth et al. laid the basis for the present studies. They showed that repair of large meniscal lesions by fibrocartilage can be achieved by implantation of a porous polymer in the connecting defect.^{29,30} Although several procedures discussed earlier result in improved healing of the tear, healing in general takes place by ingrowth of fibrous tissue not resembling normal meniscal fibrocartilage, which can also be observed in regenerated menisci. This fibrovascular scar tissue will have deviating biomechanical properties and

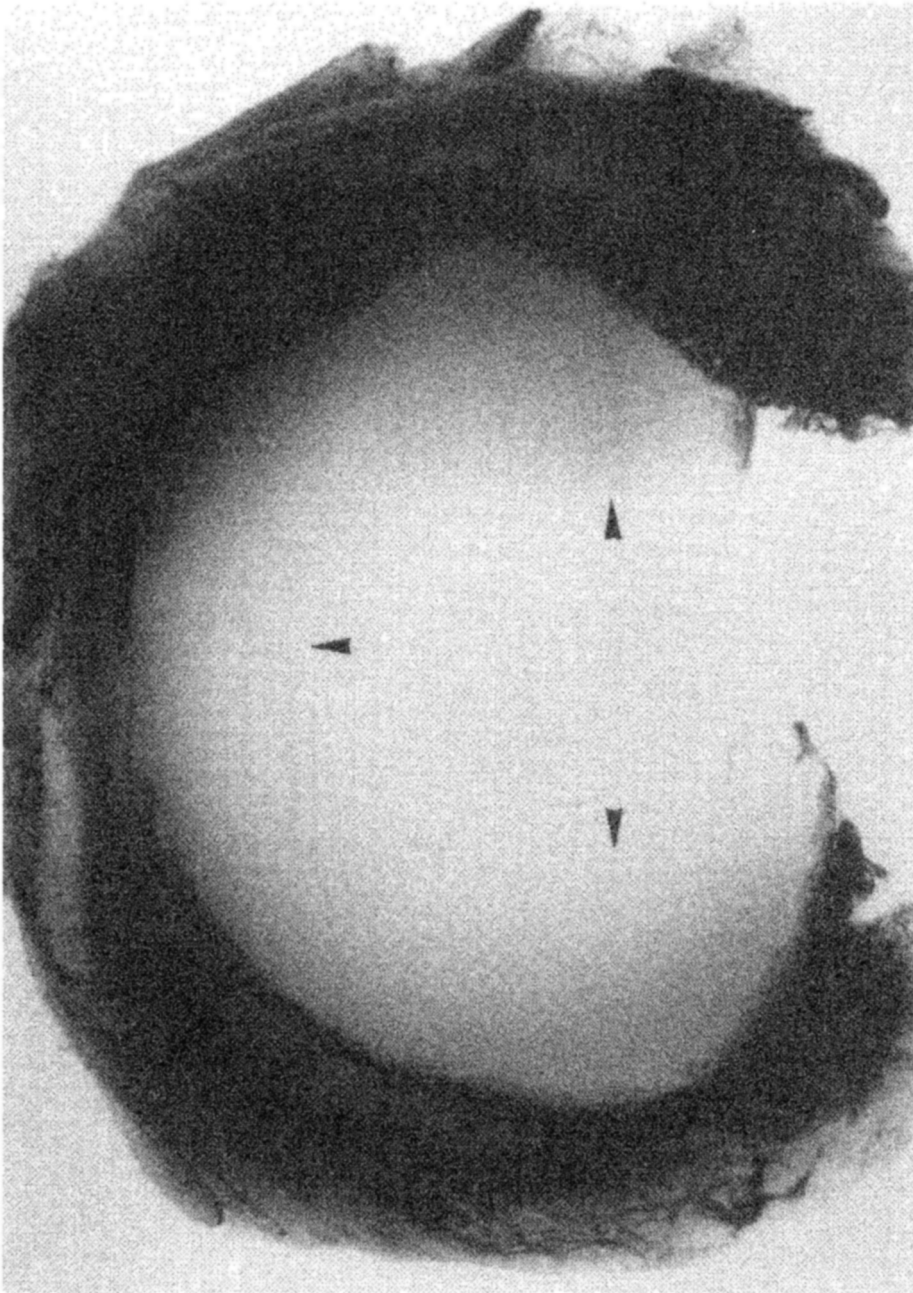


FIGURE 1. Vascularity of a normal meniscus. Only the periphery of the meniscus is vascularized. Arrows indicate the central rim ($\times 5$).

cannot be expected to function adequately in the long term.^{24,31} Repair of meniscal lesions by fibrocartilage is not frequently encountered but has been observed in spontaneously healed meniscal lesions in the rabbit,²⁶ after application of a fibrin clot or glue^{27,28} and implantation of a porous polymer.^{29,30} Sometimes a meniscus is too severely damaged to be treated by a partial meniscectomy or meniscal repair. In this case, a total meniscectomy or replacement of the meniscus by a prosthesis or allograft are the two alternatives. The results of total meniscectomy, as discussed earlier, are poor, making replacement an attractive alternative. Until now, several methods have been applied,

TABLE 1
Species Used for *In Vivo* Studies
of Meniscal Repair or Replacement

Subject	Meniscal replacement	Meniscal repair
Human	Rare	Numerous
Primate	Unknown	Unknown
Canine	Frequently	Frequently
Goat	Frequently	Rare
Sheep	Frequently	Unknown
Pig	Rare	Unknown
Rabbit	Frequently	Frequently
Rat	Unknown	Unknown
Mouse	Unknown	Unknown

but none is associated with long-term uniform success. The conclusion may be that, although allografts and meniscal prostheses may protect the articular cartilage, the results are highly variable and more research is needed.

Compared to articular cartilage, little is known about the cell biology and biochemistry of the meniscus. Studies carried out using a variety of animal species, show considerable similarities. The meniscal cells in the rabbit and human meniscus consist of fusiform cells in the superficial zone and rounded cells in the deeper zones, called fibrochondrocytes. The collagen fibers are organized into three layers: circumferentially oriented fibers, radial tie fibers and fibers woven in a meshlike fashion (Figure 2).^{22,32,33,34,35,36} These fibers can be found in all animals studied. Five types of collagen have been found in both human and animal menisci: Predominantly type I (approximately 90%) and lesser amounts of type II, III, V and VI.^{22,37} Furthermore, neural elements, fibronectin and thrombospondin are present.³⁸ All these studies suggest a large similarity between human and animal menisci but comparative studies between the species have not been carried out. To our knowledge no biochemical analysis of the primate meniscus has been carried out. One should expect a large similarity with the human meniscus. However, Le Minor³⁹ studied the morphology of the lateral meniscus in primates and found large differences between different primate species. In humans it has been shown that the meniscus size may be different for the right and left legs in the same individual.⁴⁰ Therefore, one should be careful in extrapolating results obtained in one animal species to another and maybe even from one leg to the other.

II. ANIMAL SELECTION

The subjects used for *in vivo* studies of meniscal repair or graft are listed in Table 1. In general one can say that the use of immature animals should be avoided because of the regeneration potential of the meniscus and surrounding tissues. Stone et al.^{41,42} performed studies on a collagen meniscal template. In immature pigs, resection of 80% of the meniscus resulted in spontaneous healing. Therefore, the authors concluded that in this model they were not able to determine whether or not a collagen scaffold actually stimulated regeneration of the meniscus. Canine menisci probably are the best choice. It has been shown that their regeneration potential, like humans, is unpredictable and incomplete. Also, a structure is being formed which does not resemble normal meniscal tissue and does not protect the underlying articular cartilage. The human and canine meniscus have a strong similarity concerning vascular supply, anatomy and biochemistry whereas swine, goat, sheep and rabbit menisci have anatomical features that differ from dogs and humans.^{15,16,19,20, 25,30,42-52} The rabbit has often been used for meniscus experiments, but this model may be less suitable for studying cartilage changes after meniscus surgery because the rabbit knee is very prone to synovial

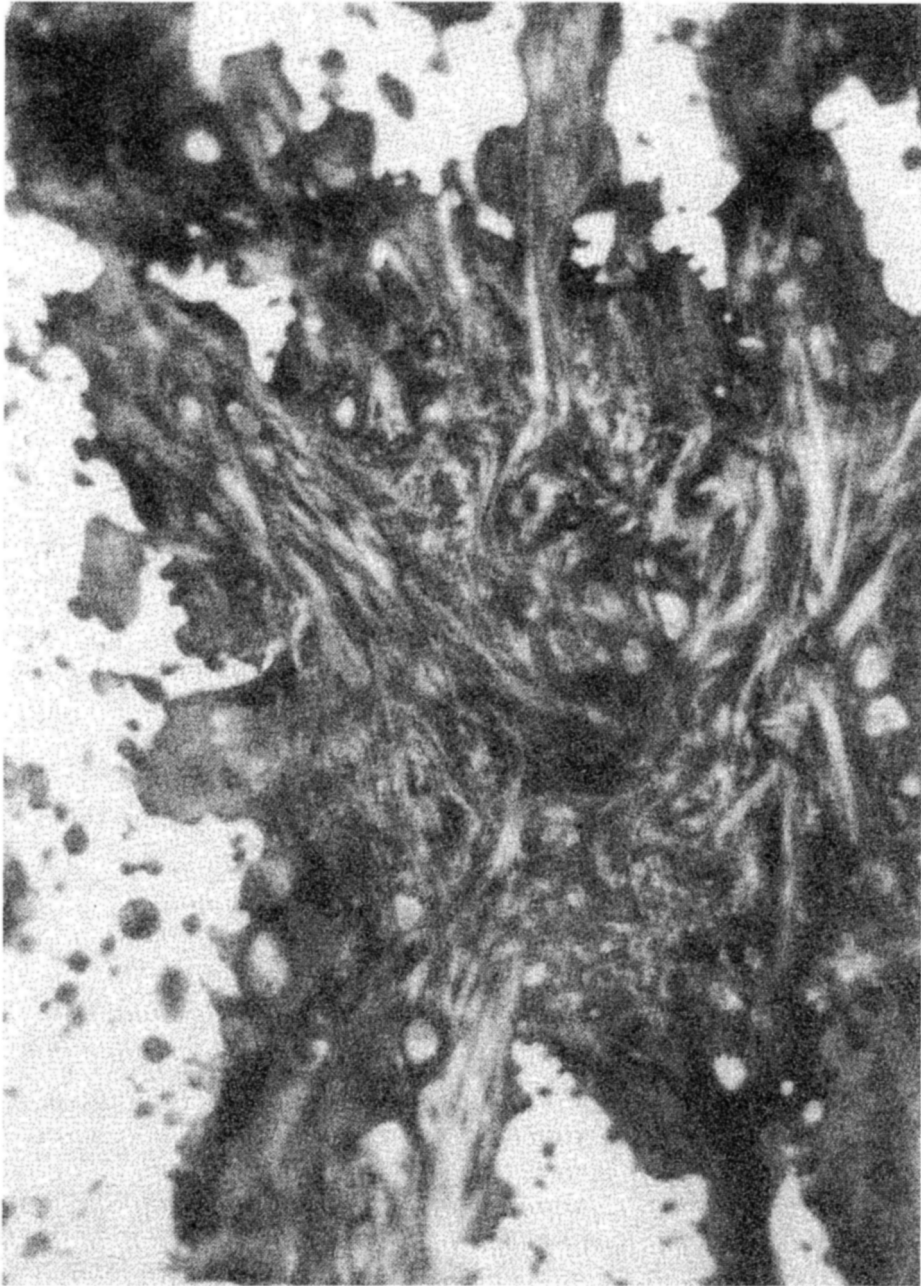


FIGURE 2. Fibrocartilage formation after implanting a porous polymer implant. Fibrochondrocytes are surrounded by an extracellular matrix of collagen fibrils.

irritation and osteophyte formation.⁵³ Knee instability after ACL resection in the rabbit causes more cartilage changes than in the dog. This may be due to the fact that the rabbit knee is more dependent on a functional ACL than, for example, a dog or human. In the rabbit the ACL is ten times stronger per unit of cross sectional area than a human ACL. Rodents like rat and mice are not suitable for meniscal repair and replacement because of their size and because of the fact that centers of ossification (ossicles) are found in their menisci. The occurrence of ossicles in human or canine menisci has been reported but they are extremely rare. Whether members of the cat family may be

suitable is questionable because there have been no reports of their use in meniscus research, and the fact that ossicles may be frequently found, as they are in the tiger.^{54,55}

III. COMMONLY USED MODELS

In fact there are three basic *in vivo* or *in vitro* models. All three have been used for meniscal repair and meniscal grafting: (1) Humans (many authors): Meniscal repair with sutures or arrows and transplantation of allografts; (2) Animals (many authors): Meniscal repair, allografts and autografts of meniscal or non-meniscal tissue, biological and synthetic scaffolds); (3) Tissue culture.^{35,36} Basic research on fibrocartilage and culture of meniscal transplants.

A. MENISCAL REPAIR

1. Humans

Many studies on meniscal repair in humans exist. Success however, can only be achieved with meniscal lesions located in the peripheral, vascular part of the meniscus. With the use of sutures (ligature, arrows and other methods) success rates of 90% and more have been achieved. Repair of the central avascular part of the meniscus has not been successful. Suggested methods are the same as those used in animals and include the creation of an access channel extending from the periphery towards the lesion, synovial abrasion or the application of a blood clot.^{24,27} Although some minor improvement in healing has been claimed, these methods are not generally accepted. Concerns and complications are the same as for animals and will be discussed below.

2. Animals

Many animal models have been employed in meniscal repair. The dog has been used for basic research,^{5,17,20,43,56} for studying repair methods,^{27,28,45–47,50,51} for implantation of a meniscal prosthesis^{46,57,58} and for meniscal transplantation.⁵⁹ The rabbit also has been a much used animal, mainly for basic research on meniscal structure,^{60,60} for studying meniscal healing and the effects of meniscectomy.⁵² Reports on meniscal repair^{26,52} and prosthetic replacement in the rabbit are frequent.^{49,53,61–69} For transplantation purposes, rabbits have rarely been used.⁷⁰ As far as we know, goats⁷¹ and sheep^{72–76} have not been used for studies on meniscal repair but only for transplantation purposes. Successful repair of peripheral meniscal lesions by suturing or abrasion of parasynovial tissue has been widely applied with good results in animal experiments using rabbits, dogs and in clinical trials on humans.^{21–23,26} This can be done by suturing using either the original suture wire or newer techniques using arrows. The basic principle is to fix the two opposing sides of the tear together, often after roughening of the surfaces.

For repair of lesions in the avascular part of the meniscus, the basic principle is to improve the vascularity of the defect by stimulating ingrowth of vascular tissue.

In the dog, this principle was realized by the creation of a radial access channel from the vascular periphery towards a lesion. Using a punch, a round channel is drilled starting at the periphery and ending in the meniscal lesion. This procedure was not very successful due to occlusion of this conduit, causing an insignificant increase in vascularity.²³

Interposition of a synovial flap between lesion and periphery has been attempted both in the dog as in the rabbit. In this technique, a synovial flap is prepared free from the capsule. Its base is left untouched and contains the blood vessels, its top is sutured in the tear. This technique does stimulate healing but repair takes place by ingrowth of fibrous repair tissue, not resembling meniscal fibrocartilage.^{24,25}

In the rabbit the longitudinal tear can be repaired by removing part of the meniscus located between tear and capsule. The central part of the meniscus can then be reattached to the joint

capsule. This method does result in healing but the cross-sectional area of the meniscus is diminished and load transmission is impaired, as with partial meniscectomy.^{23,26} Repair with fibrocartilage, resembling normal meniscal tissue, can be obtained after application of a fibrin clot in the dog.²⁷ A lesion was created in the avascular part of the meniscus and filled with an autologous fibrin clot. A combination of fibrin glue and endothelial cell growth factor appeared to provide similar results in dogs.²⁸ However, it remains to be determined whether these methods are applicable for repair of large and highly stressed lesions. Another way of promoting vascularity is by connecting the tear in the avascular part to the vascular periphery by implantation of a porous implant.^{29,30,45–51} Composites consisting of polyurethane, polylactide and carbon fibers were used for successful reconstruction of large meniscal lesions in the dogs and rabbits and repair by fibrocartilaginous tissue was observed. The key question is not only whether a technique results in healing of a tear but also if healing takes place by fibrocartilage. Healing by fibrous tissue is seen in most of the above mentioned techniques. This tissue can also be observed in regenerated menisci. It has been proven that fibrous tissue is biomechanically inferior to normal meniscal fibrocartilage and does not protect articular cartilage in the long term.^{24,25,31} Repair of meniscal lesions by fibrocartilage is not frequently seen but has been observed in spontaneously healed meniscal lesions in the rabbit,²⁶ after application of a fibrin clot or glue in the dog^{27,28} and implantation of a porous polymer.^{29,30,45–51}

B. MENISCAL REPLACEMENT

1. Humans

Replacement of the meniscus by an allograft is a new development in humans.^{73–77} The ideal patient is a person with disabling osteoarthritis of the knee who cannot be helped by a corrective osteotomy and who is too young for an arthroplasty. Large series do not exist and the long-term results are not known. Therefore, it can be considered an experimental method in humans. Concerns and complications are the same as for animals and will be discussed below.

2. Animals

As mentioned above, meniscal replacement has been carried out in a variety of animals, including humans. It is not easy to say which animal is preferable for meniscal repair or replacement. Of course, the human being is the ideal model. However, only at repeat arthroscopy can one determine the effect of repair and grafting techniques. Repeat arthroscopy cannot be done as a standard procedure in patients who do not have complaints because of the ethical aspects. Therefore, follow-up is limited to patients who develop new complaints. When an arthroscopy can be carried out, it is of limited value. The outward aspect of a graft does not provide information about tissue structure and function. For example, it has been shown that after repair of articular cartilage defects the outward appearance of such defects may be that of normal healthy cartilage whereas microscopic analysis shows fibrocartilage of inferior quality. A biopsy can be taken during arthroscopy⁷⁷ but the amount of tissue thus obtained is minimal.

Animal models seem to be essential to obtain more information. It is not known which animal is preferable for meniscal repair or replacement. When repair is performed, one needs a certain meniscus size for surgical technical reasons. The meniscus of the dog, goat and sheep appear to meet this demand. The dog's meniscus may be preferable for reasons of comparing one's own results with those provided by literature since the most cited papers are done using dogs. Using dogs, in our country, is at least twice as expensive as using goats and sheep. Lodging of dogs is more complicated and the capacity is more limited than for goats and sheep. Getting permission to use dogs for terminal experiments from ethical committees may be more difficult than it is for other animals. Other animals might be chosen as an alternative for these reasons. When meniscal transplantation or replacement is carried out, the size of the meniscus is less a deciding factor in

the choice of an animal. Technically it is possible to use the rabbit for these experiments (own experience). Our own approach in this matter is to use rabbits and goats for pilot and screening experiments and use a limited number of dogs for the key experiments.

3. Tissue Culture

Tissue culture of meniscal tissue and fibrochondrocytes was initiated by Webber et al.³⁴⁻³⁶ The advantages are, obviously, the possibility of close monitoring of cellular processes and the need for less animal experiments. In explants a small portion of the meniscus is kept in culture. Webber et al. found that explants are able to produce proteoglycans identical to those of the normal meniscal matrix. The disadvantages of these explants are the small number of cells and the limited manipulation of the cells by the extracellular matrix. Also it could not be assessed whether the cells could proliferate and synthesize a matrix. Therefore, successful attempts have been made to culture cells *in vitro* after they have been released from the extracellular matrix. This is done by enzymes such as collagenase and trypsin. In this way they could demonstrate that meniscal fibrochondrocytes are able to proliferate and synthesize a matrix. One concern of culturing meniscal cells is their changing behavior when grown in different growth media. Another problem, which may even be more important, is the process of dedifferentiation. In many studies concerning the culturing of chondrocytes taken from articular cartilage it has been shown that chondrocytes change their morphology and biochemical behavior after several days in culture and become fibroblast-like cells. This process can, to some extent, be prevented by culturing them in a 3-dimensional matrix like a collagen gel but the question remains if the cells cultured really are similar to cells in the native tissue. Our own experience with cell culture of meniscal cells is limited but we found it to be difficult, time-consuming and we had little success using this method. Certainly this method will have great value in screening toxicity of biomaterials but maybe other cell lines than fibrochondrocytes can be used. A very special application for the use of *in vitro* techniques is culturing meniscal allograft transplants.⁹⁷ They can be kept in a nutrient medium for about 2-3 weeks without loss of viability, during which period the appropriate patient can be selected and prepared. Transplant risks like disease transmission can be avoided by testing the transplants during this period. It has been suggested that culturing can reduce the antigenic potential of meniscal tissue but rejection is not a problem in reality.

IV. EVALUATION METHODS

A. GROSS MORPHOLOGY

The morphology of the implant or graft is the first method for evaluating its success. Clearly, visible destruction is an indicator for failure. Apart from implant and graft, the appearance of the articular cartilage is important. Visible fibrillation, a non-glossy appearance or even gross destruction is indicative of failure. When more subtle degenerative changes of the articular cartilage have to be detected, Meachim's test can be used.⁷⁹ In this test the articular cartilage is pencilled with India ink which fills any irregularities in the articular surface, thus making them visible. One can detect minimal changes with the use of a magnifying-glass or stereomicroscope. Measurement of the size of meniscal transplants is important because shrinkage of grafts is one of the main reasons for failure.^{73,76}

B. HISTOLOGY

The next step is to prepare the meniscus and articular cartilage for histologic examination in order to investigate the results on a cellular level. After fixation of the tissues, they are embedded in paraffin or in plastic and thin slices are cut which can be stained. Several stains are available and are used depending on personal favor or on the specific feature one wants to see. The tissues

and cells can be studied using ordinary stains like Giemsa or hematoxylin and eosin, but many other stains are suitable. For studying the cartilage-containing part of the tissue, a stain like toluidine-blue may be useful. It provides the proteoglycans in cartilage and fibrocartilage with a red color thus distinguishing these tissues from fibrous tissue. Microscopic sections taken from the articular cartilage are useful to detect minimal degenerative changes as indicated by a decrease of proteoglycan content, cloning of articular cartilage cells, fibrillation and other changes. Immunohistochemistry is a special technique using antibodies. These antibodies can detect specific cells or tissues and many are available. We have used them to show the collagen types present in meniscal repair tissue.⁵⁰ This technique is technically demanding, time consuming and relatively expensive. Polarized-light microscopy is useful in highlighting collagen fibrils.

C. MECHANICAL TESTING

Mechanical testing should be performed whenever possible. Tensile testing and deformational testing can be performed. Mow and others^{53,66,68,80} have used this technique which will answer one of the most important questions: Is the implant or graft biomechanically comparable to a normal meniscus? Numerous studies have shown that the cartilage-protecting effect of a meniscus is dependent on its biomechanical behavior. Using biomechanical testing methods, it has been found that the meniscus is a very complex structure. Its tensile and deformation behavior not only vary with location but also vary with direction. A strong correlation between the tensile properties and the collagen architecture exists. A greater degree of collagen fiber bundle orientation causes superior tensile strength and stiffness. Under compression meniscal tissue behaves as a viscoelastic tissue and its behavior makes it functionally a highly efficient shock absorber. The extracellular matrix (proteoglycans and glycosaminoglycans) of the meniscus varies continuously across its width in a manner consistent with increased compressive loading.⁸⁰ Shear tests have shown that the meniscus is anisotropic in shear, which can be explained on the ultrastructural basis. Not only the material properties of the meniscal tissue are important. The mechanical behavior of a meniscus is also highly dependent on the size, the attachments, the alignment of the knee and these factors must be taken into account.^{77,81,82} Because of the high complexity of measuring the impact of all these factors on the knee joint, most authors use computer models for finite element analysis.

D. OTHERS

Arthroscopy can be a useful tool for the gross evaluation of an allograft or a graft.^{47,76,77} It can provide data like synovial irritation, shrinkage, tearing, fraying and gross degenerative changes within the knee joint. In humans repeat arthroscopy after an operation is only done when new problems arise, whereas in animals it can be done at regular intervals. The big advantage of this procedure is that more follow-up data can be collected without the need to sacrifice the animal. However, minor changes and especially the processes going on inside the meniscus remain obscured. We have tried arthroscopy in the dog and think that technically, the procedure is easy to perform. Most likely, arthroscopy can be performed in other large animals too.

A biopsy at the time of arthroscopy can be useful to get insight in the processes inside of the tissue without killing the animal. The amount of tissue that can be removed without destroying the allograft or implant is limited. Therefore, no conclusions can be made concerning the structure as a whole.

Enzyme histochemistry can be used to test cell viability.⁷⁷ Mitochondrial enzymes (NADH-tetrazolium-reductase, alpha-glycerolphosphate oxidase), lysosomal enzymes (acid phosphatase, alphanaphthylacetate esterase) give a biochemical impression of cell viability and degeneration of cells. Uptake of radioactive sulfur by the cells has been used as an alternative method to test cell viability in the meniscus.^{83,84} Antigenic characteristics of cells and tissues and the amount of cell proliferation can be detected using monoclonal antibodies.⁷⁷

Walking analysis can be useful to assess quality of gait and joint stability.⁸⁵ Also it can be used to study if the animals favor a joint with a certain implant compared to a joint with another implant, thus indirectly comparing several implants. This method provides information in a non-invasive manner and can be repeated as many times as desired. The disadvantage lies in the fact that this method provides only indirect information about the implant or graft.

V. APPLICATIONS OF THE MODELS AND FUTURE DIRECTIONS OF RESEARCH

A. EVALUATION OF THE PATHOPHYSIOLOGY AND NATURAL HEALING PROCESS OF MENISCAL INJURY

The pathophysiology of meniscal injury has been described by several authors.^{18,31,86,87} Several types of tears exist. Tears in the medial meniscus are far more common than tears in the lateral meniscus, but the same type of tears can be encountered in either compartment. Most classifications used for meniscal tears are based on the direction of the tear. Tears in one direction are classified as: (1) Horizontal: These tears are also called horizontal cleavage lesions. This type of tear may be the result of abnormal compression forces as can be encountered in a primary varus deformity. It occurs at a later age thus causing less osteoarthritis at an early age. The lesion is caused by a degenerative process within the fibrocartilage, possibly because of a breakdown in nutrition. A decrease in the collagen/chondroitin sulfate ratio is seen at the site of the lesion, caused by an accumulation of mucopolysaccharides.⁸⁷ The femoral condyle sinks into the meniscus and extrudes the periphery causing pain, swelling and tenderness over the joint line. The degenerative changes inflicted on the femur are more pronounced than on the tibia. (2) Longitudinal: These tears are directed along the length of the meniscus and are traumatic in origin. It is more harmful because it is a lesion sustained in young patients thus causing early osteoarthritis. In case of a displaced longitudinal tear the articular cartilage is particularly susceptible to destruction. (3) Radial: These lesions are directed outward from the meniscal periphery starting at the central rim of the meniscus. In contrast to horizontal and longitudinal lesions, radial tears disrupt the circumferential collagen fiber structure. For this reason, meniscal function is more affected by radial tears.

Smillie⁸⁷ mentioned the main features of a meniscal lesion. Most importantly the symptoms are intermittent. The patient is older than the age of 30 and younger than 57. A history of trauma may be absent in case of a cleavage tear but is often present in case of a longitudinal tear. Local pain is present in the joint line. There is a feeling of giving-way and instability. Lack of extension (locking) of the knee is present when a flap is interposed between the femur and tibia. Effusion is not a specific symptom.

There's no doubt that the meniscus has a limited healing potential. King's experiments in 1936 set the biological limitations of meniscal healing. He showed that for meniscal lesions to heal, they must communicate with the peripheral vascular area of the meniscus.¹⁸ Later it was established that only the outer 10–25% of the human and dog's meniscus, throughout its attachment to the joint capsule, is vascularized. This explains why there is no tendency for healing when tears occur in the avascular central part of the meniscus.^{19,20} As a result only lesions limited to the vascular periphery can be repaired adequately by simple suturing. For lesions situated in the avascular central part of the meniscus no healing is observed. The microscopic appearance of meniscal tears is similar regardless of the type of tear. A repair reaction including hemorrhage, vascular invasion and formation of meniscal tissue is seen in the vascular periphery of the meniscus. Whether a perfectly normal microscopic structure is formed is not known. It may be that, especially in case of a healed radial lesion, the circumferential collagen fibers are never fully restored to normal.⁴⁷ Although tears located in the central avascular part of the meniscus do not heal spontaneously, healing can be achieved by several methods. The basic principle is to improve the vascularity of the defect by stimulating ingrowth of vascular tissue. Although several procedures do result in improved healing

of the tear, healing in general takes place by ingrowth of fibrous tissue not resembling normal meniscal fibrocartilage, which can also be observed in regenerated menisci. This fibrovascular scar tissue will have deviating biomechanical properties and cannot be expected to function adequately in the long term.^{24,25,31} Repair of meniscal lesions by fibrocartilage is not frequently encountered but has been observed in spontaneously healed meniscal lesions in the rabbit²⁶ after application of a fibrin clot or glue^{27,28} and implantation of a porous polymer.^{29,30} (Figure 3) After partial or total meniscectomy remodeling and regeneration has been observed both in animals as in humans.^{35,36,44,85,88} Remodeling and regeneration are thought to be caused by migrating cells from the synovium or a proliferation of surrounding meniscal fibrochondrocytes, possibly stimulated by the presence of a fibrin clot and growth factors. However, these phenomena are not predictable and usually a smaller meniscus is being formed. This meniscus consists of tissue not resembling normal meniscal fibrocartilage. This tissue is biomechanically inferior to normal fibrocartilage and osteoarthritis is not prevented.

B. EVALUATION OF ARTIFICIAL SUBSTITUTE OF MENISCUS

1. In General

Perhaps the most attractive option for the repair and replacement of a torn meniscus is the use of artificial materials. In contrast to biological grafts they do not transmit diseases like hepatitis, AIDS and others. Sterilization of the implant will be easy, whereas in biological grafts sterilization will affect its properties. Also the limited availability of biological grafts is overcome since artificial substitutes can be made in unlimited numbers. The problems of storage do not exist, and many meniscal shapes and sizes could be obtained. Work on the use of artificial substitutes is not new but the older concepts of implanting a solid material have proven to be unsuccessful. Therefore, newer designs are based on porous materials which allow ingrowth of tissue.

Two groups of materials can be distinguished, nondegradable materials and degradable (or resorbable) materials. Theoretically, it seems unlikely that nondegradable materials will ever approach, both structural and biomechanically, a normal meniscus because of its enormous complexity. A degradable material ultimately forms a structure composed of normal body tissue. Therefore, it seems likely, although not proven, that a degradable material will result in a meniscal replica which most strongly resembles a normal meniscus. The rationale for the use of resorbable scaffolds is often based on the work of Yannas and Burke who were the first to use artificial skin.⁸⁹ The scaffolds must fulfill the following demands and more demands may be added in the future (several of these demands also apply for nondegradable implants).^{42,45–51,89} (1) They must be biocompatible and should not evoke a foreign-body reaction. One should realize that the biocompatibility of any material used not only depends on its composition but also on its application. For example, the use of carbon fibers elicits a synovial inflammation in the knee whereas the same material can be used without problem in another part of the body. (2) They must be biodegradable to avoid any long-term complications. (3) Their degradation rate must be slow enough to permit ingrowth of pro-meniscal tissue and transformation into fibrocartilage. We have seen that quickly degradable implants do not give rise to the formation of fibrocartilage. (4) The pore size of the biomaterial must be optimized because it controls the speed of ingrowth into the implant and the amount of fibrocartilage formed. (5) They should have the same size and morphology as the normal meniscus, or acquire these features as soon as possible since incongruous grafts will lead to degeneration of the meniscus or the articular cartilage.⁴⁰ (6) The scaffolds should provide a tissue having identical biomechanical properties as a normal meniscus as soon as possible thus avoiding or retarding degenerative changes of cartilage. (7) The attachments must be anatomical. It has been shown that a non-anatomic position of the attachments leads to osteoarthritis. For this reason, allografts are transplanted together with the bone blocks at the attachment sites.

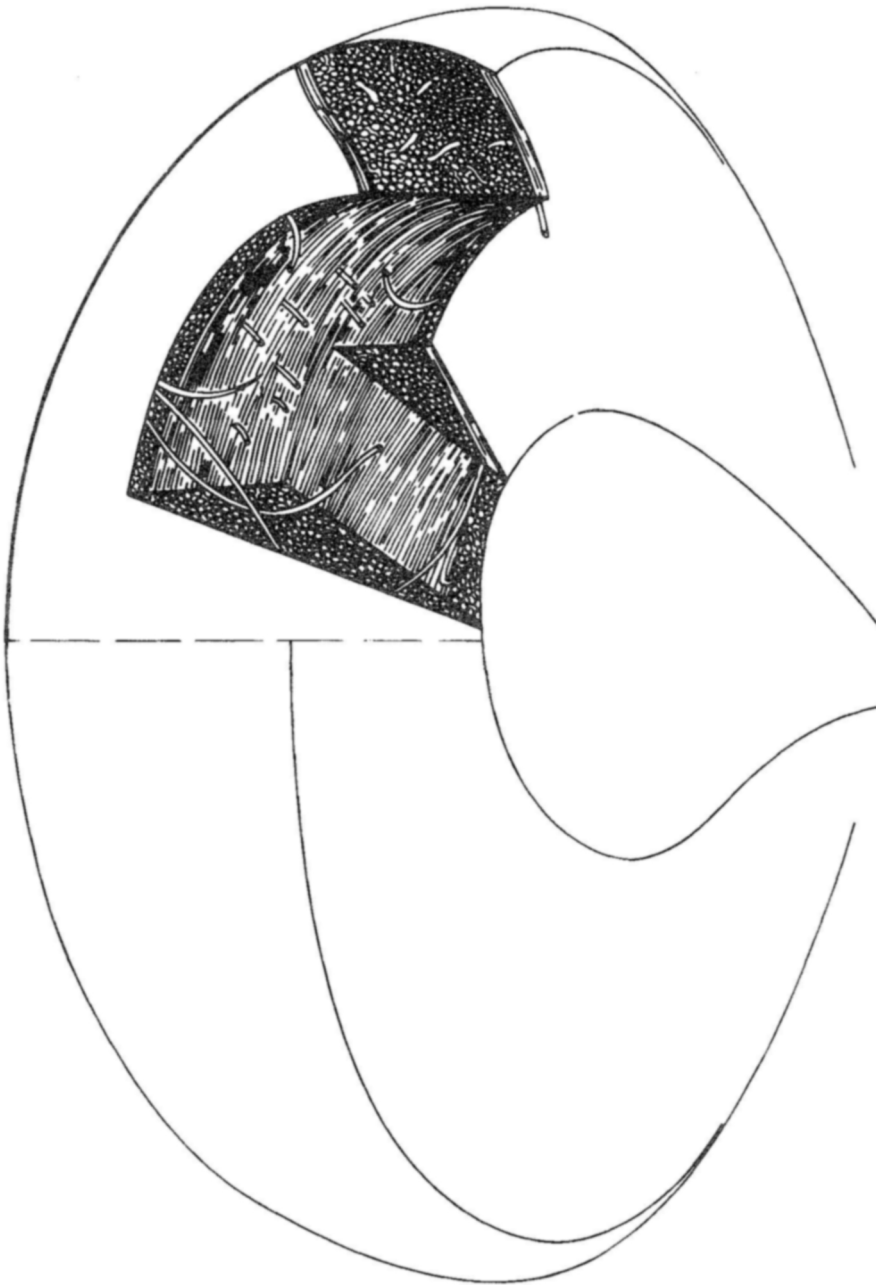


FIGURE 3. The complex meniscal structure. The main collagen fibrils are oriented in a concentric configuration interwoven by radial tie fibers.

2. Nondegradable Implants

A Teflon net prosthesis in the dog provided one of the first encouraging results in using an artificial meniscus.⁵⁷ A Teflon net was folded into a cylinder and sutured to the joint capsule. Although cartilage degeneration was not prevented, and was observed as soon as three months

post-operatively, it was less severe compared to meniscectomy. Histologically, Teflon allowed ingrowth of tissue into its interstices; it also elicited a clear inflammatory response. After nine months cartilage cells could be observed. Clearly, the induction of cartilage-like tissue was a positive finding. It is speculated that joint instability, improper fixation and adhesion of the graft to the popliteal tendon thus impeding knee motion are responsible for the joint degeneration and that Teflon as a material is superior to previously used materials. However, the early degenerative changes make Teflon not used in this form unsuitable for meniscal replacement.

Wood et al. used a prosthesis of concentrically carbon fibers ensheathed by woven polyester fibers to replace the rabbit's meniscus with disastrous results.⁶⁹ The prosthesis was secured using a transosseous tibial tunnel and it was sutured to the capsule. The carbon fibers fragmented staining the synovium black. Osteophytes were present in all knees and the menisci showed lateral displacement. There was no invasion of fibrous tissue and the implants were encapsulated instead of being incorporated into the surrounding tissue. A marked inflammatory response was shown around the polyester fibers. It was concluded that this prosthesis in its present form did not work. It appeared that this prosthesis showed the following negative features: (1) Carbon fibers may be compatible for certain applications, but they were not in the knee joint. (2) The prosthesis was not porous and its fixation not rigid enough. A knee with intact menisci can store more energy than a knee with the meniscus removed. Joint stiffness increases after meniscectomy. The biomechanical effects of a Dacron prosthesis with a polyurethane coating was tested in rabbits.^{53,66-68} Knees with a prosthesis showed lower energy storage than sham operated knees and energy storage was similar to the meniscectomy group. After three months cartilage changes were found in 70% of the prosthesis group, compared to 100% in the meniscectomy group.

The cartilage changes on the femur were comparable for both groups; the tibia was less affected in the prosthesis group. Synovial changes with fibrosis were noted in 90% of the prosthetic knees but no foreign body reaction or loose Dacron particles were found. It was concluded that the prosthesis had a cartilage-protecting effect but the biomechanical behavior of the knee joint was similar to meniscectomy.

Partial ingrowth was seen in 50% of the prosthesis and fibrocartilage had not formed. The authors think that an improper size and inferior biomechanics of the prostheses may be responsible for the failures. Therefore, a new series of prosthesis was designed. These prostheses had better *in vitro* biomechanics than the previously tested prosthesis. A polyester (Dacron), a PTFE (polytetrafluoroethylene) implant with a polyurethane coating on the upper surface and an uncoated PTFE implant were implanted in rabbits.⁶⁴ The PTFE prosthesis had compression values that matched the normal meniscus in contrast to polyester. After three months cartilage softening and osteophyte formation was found in all groups. Ingrowth of tissue was not complete and fibrocartilage was not found. The uncoated PTFE prosthesis, which had mechanical properties most closely resembling a normal meniscus, lost its shape and was prone to wear causing debris. Its effects on the cartilage was similar to meniscectomy. The polyurethane coating helped to conserve the shape and material of the implant. The polyester prosthesis showed little ingrowth, probably because of too little porosity. The coated PTFE prosthesis gave the best overall results. All prostheses elicited a synovial reaction and osteoarthritis was not prevented. The load-relaxation characteristics of joints with implanted prostheses were better than those of meniscectomized knees, in contrast to previous studies. This is explained by a better anatomic position and better sizing of the prosthesis.

The same authors also studied the effect of a meniscal prosthesis in an unstable knee after resection of the anterior cruciate ligament.⁵³ It appeared that no protective effect of a prosthesis is present in an unstable knee. Therefore, just as applies for meniscal repair, a stable knee joint seems to be a prerequisite for success. A combination of an artificial prosthesis of Teflon combined with a biological periosteal implant in rabbits did not improve results.⁶¹ All grafts had changed in shape and were extruded towards the periphery of the joint. The cartilage no longer was covered and protected. Fibrocartilage was not formed and the meniscus failed to carry out meniscal function.

3. Degradable implants

A collagen-based scaffold has been reported to give an excellent meniscal replica in swine and canine.^{41,42,85,90–94} Tissue from bovine Achilles tendon is formed into meniscal disks. These disks are infused with glycosaminoglycans in amount approximating those found in human menisci. These scaffolds are reported to be non-toxic because they allow the ingrowth of fibrochondrocytes. When implanted in the knee joint of immature pigs excellent regeneration of the meniscus was reported, which is not surprising because meniscal regeneration in immature animals is present anyway. After implantation in mature dogs, 63% of competent meniscal regeneration was noted compared to 25% for control menisci. After a maximum follow-up of 12 months no significant difference in the gross appearance was seen between joints that had received collagen implants and those that had not. Synthesis of proteoglycan by the templates had normalized by nine months. Histologically, an excellent resembling meniscal replica was formed. Implant material remained visible in the regenerated menisci at 12 months.

One restriction of using this technique is that the scaffolds are sutured to a remaining peripheral rim of meniscal tissue. In reality, a remaining peripheral rim may not be present. Therefore, at least in the animal, this technique has proved to be suitable for the partial replacement of a meniscus rather than for total meniscal replacement. Our own experience in artificial meniscal substitute is done with resorbable materials in the dog, rabbit and goat (Figure 4).^{30,45–52} The first attempt was to repair meniscal lesions using a combination of carbon fibers and polyurethane-poly lactide. These grafts appeared to be unsuitable because of carbon particle induced synovitis. Later, mixtures of polyurethane and polylactide were used to form several porous implants. Implants initially became filled with vascular fibrous tissue and displayed a mild foreign body reaction consisting of polynuclear giant cells, some macrophages, and lymphocytes. After two months the fibrous tissue filling the implants became transformed into metachromatic avascular fibrocartilage strongly resembling normal meniscal tissue. The repair tissue initially consisted of fibrous tissue containing type I collagen. Later, this vascular fibrous tissue was transformed into avascular fibrocartilage. Both type I and type II collagen, the major collagen types of normal meniscal fibrocartilage, could be detected in this newly formed fibrocartilage. In control defects, which were filled with vascular fibrous tissue without fibrocartilage, only type I collagen could be detected. Type II collagen was never found. This reaction took place in all implants used, based on porous physical mixtures of polyurethane, polylactide, and caprolacton. There were, however, considerable differences in ingrowth and fibrocartilage formation among the implants used. In contrast to the transformation of fibrous tissue into fibrocartilage, as can be observed in implants, empty control defects and synovial flap defects were filled with vascular connective tissue which did not transform into fibrocartilage. Roughly, two-thirds of the longitudinal tears could be repaired using this method.

It is well known that the implant's physical structure can be of great influence on its biological behavior. It has been shown for several tissues that it not only alters the rate of tissue ingrowth but also the degree and type of differentiation of ingrowing tissue, thus determining the ultimate type of tissue formed. Therefore, implants with varying pore structure were tested for their biological behavior in rabbit menisci. It appeared that tissue ingrowth was optimal in two large pore implants (macropores of 150–250 and 250–500 μm) whereas small pore implants (macropores of 50–90 and 90–150 μm) remained partially empty up to one year postoperative. Capsule formation and the foreign body reaction was severe for the small pore implants whereas this occurred to a lesser extent in the two large pore implants. Fibrocartilage formation, as assessed by morphology and antibody labeling for type I and type II collagen, was observed in a similar way in all implant types. It was concluded that for optimal ingrowth and incorporation of partial or total meniscal prostheses, macropore sizes should be in the range of 150–500 μm .

After it was shown that small defects could be repaired by porous polymers, whole menisci were made of the same porous polymer and implanted in the dog. Sixteen knees received a prosthesis. The tissue reaction was essentially identical to the one seen in small implants used for

meniscal repair. In the first six knees the prostheses were secured using single sutures which were only pulled through the anterior and posterior prosthesis horns. At sacrifice four of these prostheses appeared to be dislocated due to tearing-out of the sutures. Therefore, the following prostheses were secured using two sutures running longitudinally through the entire prosthesis. Of the remaining 10 prostheses only one dislocated. The short-term implants had a connective tissue appearance, similar to dislocated implants. They were light-brown of color and had a soft consistency. After three months the prosthesis had a yellowish glistening appearance, and had a firm consistency. A rim of hyaline-like neocartilage had formed at the prosthesis' inner margin.

After an initial ingrowth of vascular fibrous tissue containing type I collagen only, the prostheses became filled with fibrocartilage strongly resembling normal meniscal fibrocartilage, containing both type I and II collagen. Degenerative changes of articular cartilage were present in all meniscectomized control knees. Moderate fibrillation of cartilage seen at eight weeks progressed to severe destruction exposing the subchondral bone after 20 weeks. Degeneration in association with a dislocated prosthesis was comparable to meniscectomy and was more severe when follow-up periods were longer. Degeneration associated with well incorporated prostheses was frequent, although less severe than seen after meniscectomy or dislocation. Intact cartilage was seen in five knees. In the remaining six knees, varying degrees of cartilage destruction were seen, although exposure of the subchondral bone did not occur. In two of these six knees it appeared that the drill holes were located in the central part of the tibial cartilage instead of in the eminentia. This surgical error may have contributed to the cartilage damage. Cartilage degeneration was not related to the length of follow-up or formation of fibrocartilage inside of the prosthesis. Tibial plateaus were more frequently and more severely affected than femoral condyles. Although less severe than seen after total meniscectomy, cartilage degeneration was frequent, possibly because tissue ingrowth in the prostheses occurred too slowly, because the size of the prosthesis was incorrect and due to surgical error. It was concluded that porous polymers can be useful for replacement of the meniscus, provided that chemical and physical properties are optimized.

C. EVALUATION OF BIOLOGICAL GRAFT

1. Autografts

Autograft tissue is harvested elsewhere in the body and is used for a meniscal substitute. The advantage of autograft is its availability and the absence of immunological reactions. It is thought that non-meniscal tissue can transform into meniscal tissue when implanted in the knee. In both humans and sheep the meniscus was replaced by the infrapatellar adipose body.⁷³⁻⁷⁶ Although there were no adverse effects, all grafts resulted in a soft tissue not resembling a normal meniscus. Also the size of the grafts decreased in time. An alternative method is to use a strip of quadriceps tendon which is sutured in place.^{40,72,82,90} This method is in development and clinical results have to be awaited.

2. Allografts

Allograft menisci comes from a different animal of the same species. Allografts are the state of art to replace a complete meniscus. Both human and animal trials have proven that transplantation of a meniscus is technically possible.^{59,70,77,78,83,92} Theoretically, this method provides for a replacement meniscus that is as much identical to the original one as it possibly can be. Its disadvantages have been mentioned before and include the danger of disease transmission, the limited availability and storage time and problems of sterilization. In theory, allograft menisci could give rise to immunological reactions. Although the meniscus certainly has immunologic potential, rejection is not seen in practice. However, when the attachment sites are transplanted using bone blocks, the bone can elicit an immunological response in the recipient. In practice this does not result in



FIGURE 4. Degradable polyurethane implant after 12 weeks' implantation in the dog. The implant is covered by fibrocartilage. Arrows indicate the central rim.

rejection of the graft.^{73,82} It is not exactly known whether the transplant must contain living cells or if it should only serve as a template which is repopulated by cells from the patient.

When living cells are to be transplanted, the meniscus cannot simply be frozen as this will kill the cells. Two options are available. First, fresh tissue can be used. This method has considerable logistical problems. The tissue can only be stored for a limited time. The main problem of fresh grafts is the danger of transmitting disease. The donor may be infected without yet being seropositive.

The risk of HIV transmission from soft tissue allografts in appropriately screened donors is about one in one million.⁹³ The second method is to use cryopreserved menisci. These menisci are frozen in a slow and controlled way using cryoprotectants. A small percentage (10–30) of the original cells will survive and preserve the mechanical properties of the transplant immediately after transplantation. Lyophilized menisci only consist of a collagen structure. The cells and proteoglycans are removed. A decrease in size was noted over time and the results are worse than for fresh and fresh-frozen grafts.^{76,82} In contrast to lyophilized grafts, deep frozen menisci do show healing to the periphery but showed little revascularization or remodeling when implanted in sheep.⁷⁴ Cryopreserved menisci in dogs all healed to the periphery. Cellularity and metabolic activity returned to normal after six months. Damage to the tibial cartilage was present although it was less than after meniscectomy.⁹² Menisci treated with glutaraldehyde have been used in animals but were not successful.⁵⁹ All knees showed an effusion and the menisci did not demonstrate healing to the periphery. Cellular repopulation of allografts was studied by Arnoczky.⁸³ He found that the deep frozen meniscus was repopulated by synovial cells from the recipient. However, the lack of repopulation of the meniscal center and the loss of the normal collagen orientation was a matter of concern. In humans fresh^{73,76} or cryopreserved⁷⁷ allografts have been used with good results. This procedure can be done in conjunction with a reconstruction of the anterior cruciate ligament since meniscal transplantation in an ACL-deficient knee will result in failure. Allograft selection is a big problem. Especially, determination of the size is difficult. There is a large variation in size among patients and there is considerable difference in size between contralateral lateral and medial menisci. MRI and CT scans underestimate or overestimate the meniscal size an average of 2.8–4 mm and it is well known that improper sizing will deteriorate the results. If a meniscus is too large, the periphery may be trimmed but the circumferential collagen fibers must be kept intact. If the graft is too small it should not be used. The indications for transplantation are not well defined. Theoretically, the best time for transplantation would be immediately after meniscectomy because cartilage degeneration will be minimal and the reduction of progression of the wear would be maximal. However, clinically the authors consider patient age, knee stability, the degree of osteoarthritis and pain. A painless patient who does show cartilage degeneration on the X ray is not a candidate for transplantation. Also patient age is important. The majority of authors operate on patients who are in their twenties to forties and who are too young for a total knee arthroplasty. This indicates that meniscal transplantation still is in an experimental phase. Clinically fresh and cryopreserved allografts can heal to the periphery but their cartilage protective effect in the long term yet has to be determined.

3. Xenografts

Xenograft meniscal tissue (menisci from one animal species implanted into another species) does not seem to be a realistic option. Although rejection probably will not be a problem, there are large differences in meniscal sizes and composition among different animals. Since size and anatomy are crucial, this will result in failure.^{81,96}

4. Cell-Seeding

Cell seeding of artificial scaffolds may accelerate ingrowth of fibrocartilage. This experimental technique has been used successfully for the repair of defects in articular cartilage in animals. Cells are harvested, released by enzymes and kept in appropriate culture media until they are implanted. Webber^{35,36} has proven that meniscal cells can survive in tissue culture and are able to migrate. One of the problems could be the dedifferentiation process of the cells. From articular cartilage cells it is known that they lose their phenotype and become fibroblast-like cells when kept in culture. This process can be prevented when the cells are grown in a three-dimensional matrix such as a collagen gel. It is unknown if fibrocartilage cells show the same process of dedifferentiation.

Coating of scaffolds with adhesion molecules such as fibronectin or chondronectin, chemotactic factors or with growth factors may increase and accelerate cellular ingrowth and fibrocartilage formation. In tissue culture, fibrochondrocytes do respond to these factors, thereby enhancing ingrowth into a matrix.

VI. SUMMARY AND CONCLUSIONS

The meniscus is an important structure of the knee joint and is essential in preventing osteoarthritis. Every effort to preserve this structure should be made. When this is not possible, meniscal replacement is a good alternative. Allografts can provide good results in the short term but have important disadvantages. Synthetic grafts have, at least in theory, fewer disadvantages but until now none has proven to be able to match the results of allografts. Future research will have to improve their qualities in term of biomechanics, induction of fibrocartilage and others. Several animal species can be used for meniscal research but the dog may be the preferable animal.

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18 Animal Models of Osteoarthritis

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I. INTRODUCTION

The development of osteoarthritis involves a complex interaction between environmental, biological (including sex and genetic contributions), and mechanical components and, at least in the latter stages of disease, involvement of all the tissues surrounding the joint.^{1,2,3} Human tissues usually represent end-stage disease, therefore, animal models are critical to understanding the disease, and

validating interventions.^{1,4} This is a practical overview of the osteoarthritis models, their advantages and disadvantages, and is not comprehensive. Readers are referred to earlier reviews that outline relevance of models to human disease.^{1,4,5}

All models have limitations, so first the questions being asked need to be clearly defined. For comparison to human disease, histologic characteristics of osteoarthritis that develop naturally in three commonly used species are illustrated in Figure 1. Each species demonstrates gross similarities and show subtle, or not so subtle, differences that should be kept in mind. There are the usual concerns, such as, are the molecular targets the same as in humans or even present, and especially for a drug study, is the metabolism of the cartilage and of the compound appropriate? However, some questions are unique to osteoarthritis models. These are: Will joint geometry make a difference; Does treatment evaluation require progression to the same end-stage disease as the human; What is the balance between how fast the model develops and the expected treatment effects. Human osteoarthritis develops over decades, thus a sustained, modest effect might be sufficient to delay progression, but may be undetectable if it is tested in an aggressive model. In general, it is recommended that an intervention be tried in three models involving two species with at least one being a surgical model.

After the model is selected, the choice of read-outs is the next most important parameter and should be determined in advance. The method of evaluation should be appropriate to questions that are being addressed, both in terms of sensitivity and specificity, and reflective of the aspect of joint physiology that will be followed. Additional information that will be collected must also be decided at the beginning. Some currently used methods are addressed later in the chapter.

Pritzker⁴ defined availability criteria for animal models of osteoarthritis and he included the following: cost; previous research in the model (background, knowledge, and peer acceptance); ease of experimental manipulation; ease of handling; supply of animals of the appropriate age, size and sex; availability of experimental techniques for model evaluation and manipulation; anatomical and physiological attributes including sufficient joint tissue and fluids; quantitative resemblance to human tissue; weight-bearing or nonweight-bearing nature of joint; availability of matched controls; life span of animal relative to onset of disease and time-frame of disease progression; availability of nutritional and exercise history; and genetic background. To this list might be added: what phase of the process is being modeled (early-, middle- or end-stage), does bone quality and bone biology make a difference, relevance of use of immature, mature, or aged animals, and especially in rodents, what are the consequences of the presence of an open growth plate?

In broad terms, there are three types of models. They are spontaneous or naturally-occurring models, a group that includes genetically-manipulated mouse models, chemically-induced models, and mechanically-induced models. Mixed models such as combined mechanical and chemical alterations have seen only limited development. For additional discussion of early literature for many of these models, the readers are referred to older reviews.^{1,4,5-7}

II. ANIMAL MODELS OF OSTEOARTHRITIS

A. SPONTANEOUS MODELS

All vertebrate animal species develop osteoarthritis, but at different rates and sometimes with different joint distributions (Table 1).¹⁸ Spontaneous models have the advantage in that they occur naturally, but their major disadvantage is the time they take to develop. These are being expanded with transgenic models where matrix or cellular proteins that rapidly cause osteoarthritis (such as types IX and XI collagen mutations)^{20,21} are targeted, but where the phenotype is not so severe that it prevents development and growth. Mouse models will become very powerful tools for exploring the contribution of specific proteins to the osteoarthritic process and may provide accelerated models for evaluating interventions.

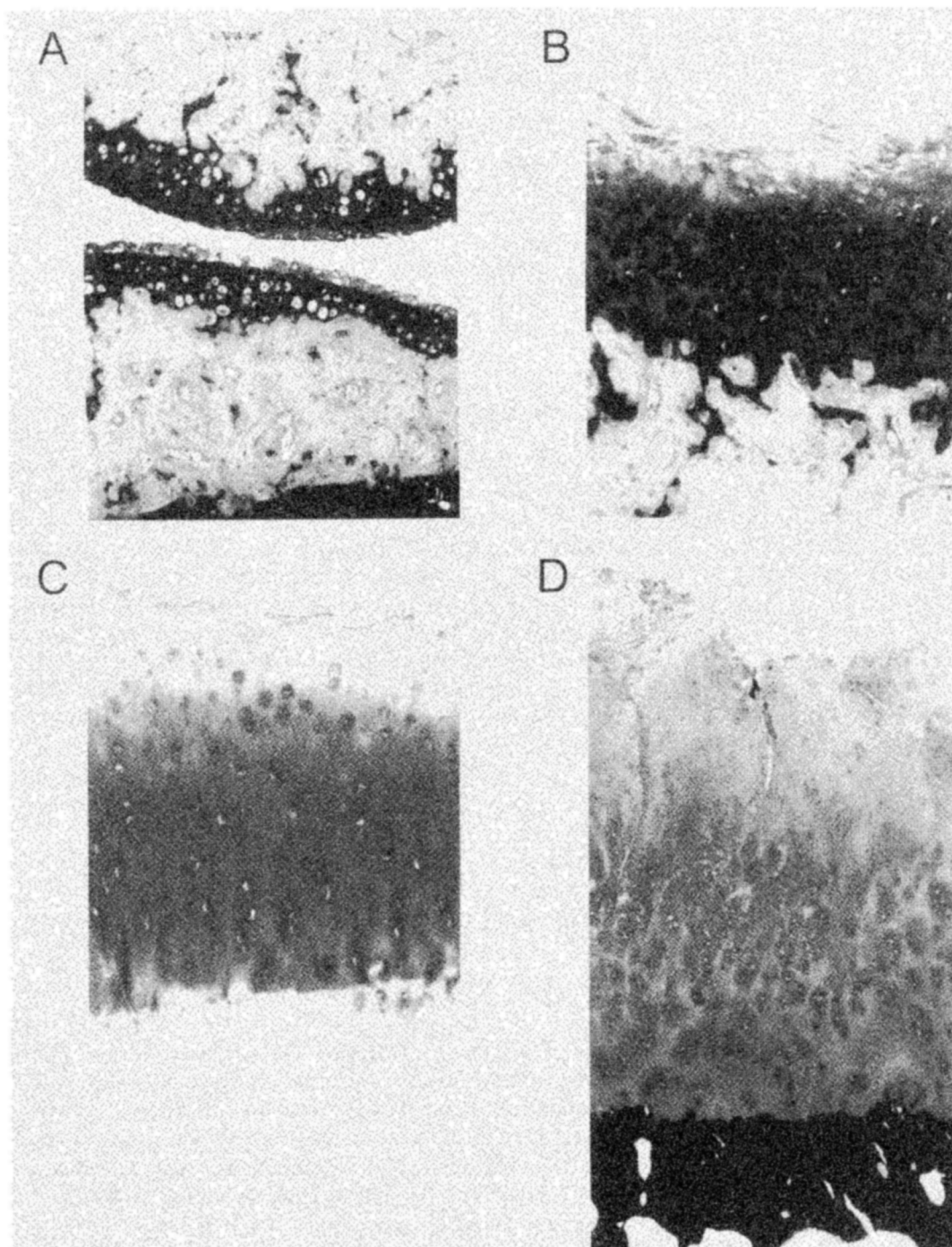


FIGURE 1. Photomicrographs from a sagittally sectioned medial portion of the knee joint of a partial medial meniscectomized mouse six weeks post surgery (A), sagittal section from the medial tibial condyle of a partial medial meniscectomized guinea pig nine weeks post surgery (B), sagittal section through the distal aspect of the medial trochlear ridge of the left stifle joint of a dog 12 weeks post transection of the anterior cruciate ligament (C), and osteoarthritic cartilage from the right tibial condyle of a 72 year old female who underwent arthroplasty surgery. Toluidine blue, original magnification 12.8 \times .

TABLE 1
Spontaneous Models

Animal	Strain	References
Mouse	C57BL	8,9
	STR/IN	10
	STR/ORT	11
Guinea pig	Hartley	12–15
Canine (hip)	Labrador retriever	16
Monkey	Macaca malatta	17
Rat		18
Hamster		19

B. ANIMAL MODELS OF MECHANICALLY/SURGICALLY-INDUCED DISEASE

Mechanical models were developed to provide an accelerated, but controlled model of osteoarthritis or, in some cases, to demonstrate the contribution of particular mechanical components or structural elements to the development of osteoarthritis (Table 2). Attempts to repair the initial alteration and potentially make the model reversible have not been done. The anterior (cranial) cruciate ligament transection model in the canine is considered the gold standard where the early, mid- and end-stage aspects of the disease have been carefully documented and eburation to bone takes 3–4 years.^{46,47} Meniscectomy is performed in several versions, and progression and extent of late changes depend on the model. Partial medial meniscectomy in the rabbit produces a mild, progressive disease.¹ The complete meniscectomy in the sheep produces rapid progression.^{42,63} The partial meniscectomy, in combination with anterior cruciate ligament resection or anterior cruciate ligament and posterior cruciate transection, also rapidly produces extensive disease in the rabbit.^{1,41,64} Extraarticular surgeries, such as varus and valgus osteotomies and muscle resection, produce disease more slowly (Table 2). Osteotomies need additional instrumentation and more operating time, and are technically challenging.

Especially in mechanical/surgical models, the animal often alters use of the opposite leg, which can cause persistent changes.⁵⁹ These changes do not progress, so the opposite limb may be an appropriate gross morphologic or histologic grading control for mid- and end-stage disease studies, but a poor control for detailed studies.⁵⁹

C. CHEMICALLY-INDUCED MODELS

Direct injection of active agents into joints has been investigated (Table 3). These models usually show dose-dependent damage and at least at low levels of damage are potentially reversible.⁷⁸ Characteristically, for the enzymatic models, the damage begins from the surface down and may be varied throughout the joint because of differential access of reagent or differences in cartilage composition. Damaged areas are then vulnerable to normal joint forces. Progression can be accelerated with exercise or, in some cases, delayed with immobilization,⁶⁸ or improved with passive motion.⁷⁶ The reagent may also damage the secondary structures of the joint and alter the joint mechanics.⁶³ Mechanical and chemical models of other joints, besides the knee, have been reported (Table 4).

D. AUTHOR-PREFERRED MODEL

If the objective is to study the etiopathogenesis of osteoarthritis, then each model has its merits with many of the models elucidating changes in chondrocyte and bone metabolism. So the model may best be decided by the requirement of the methods that are going to be applied, such as the

TABLE 2
Mechanical/Surgical Models

Models	Animal^{Ref.}	Comments
Focal defects	canine, ²² rabbit ²³	see Chapter 16
Scarification	rabbit, ²⁴ canine ²⁵	canine: nc at one year
Freezing	rabbit ²⁶	nc in 6 months; sb in 12 months
Repetitive impulse loading	rabbit, ²⁷ guinea pig ²⁸	sf, sb
Closed transarticular impact	rabbit, ^{29,30} canine ^{61,92}	sf, eb, cl, pl
Open transarticular impact	canine ³²	sf, eb, cl, pl
Tibial varus osteotomy	rabbit ³³	os, sf, cl in 34 weeks
Tibial valgus osteotomy	rabbit ^{33,34}	os, sf, cl in 34 weeks
Gluteal resection	guinea pig ³⁵	
Gluteal resection (intrapatellar ligament release)	guinea pig ³⁵	sf, cl, sb in 24 weeks
Denervation	rabbit, ³⁶ canine ³⁷	rabbit: cd, canine: nc, 64 weeks
Denervation/ACL resection	canine ³⁷	accelerated change over ACL
Partial meniscectomy (anterior aspect medial 1/3)	rabbit, ¹ mouse, ³⁸ guinea pig ³⁹	rabbit, ~70% develop sf, cl, os in 4, 8 weeks, 20–25 extensive disease
Partial lateral meniscectomy (colateral sesmoid ligaments)	mouse ⁴⁰	
Partial meniscectomy + PCL + ACL	rabbit ⁴¹	
Meniscectomy	sheep, ⁴² rabbit, ⁴³ monkey ⁴⁴	
Anterior cruciate resection	rat, ⁴⁵ canine, ^{46–48} rabbit ⁴⁹	
Posterior cruciate resection	rabbit ⁵⁰	mild focal pitting
Patellar dislocation	canine ²²	
Patellectomy	rabbit, ⁵¹ sheep ⁵²	cd, sf, eb, os, pl
Polyethylene insert	rabbit ⁵³	rapid, eb, cl, os, pl
Step-off fractures	rabbit ⁵⁴	pl, cl, os, eb
Resection of femoral condyle	rat, ⁵⁵ rabbit ⁵⁶	excess pannus
Immobilization	rat, ⁵⁷ rabbit, ^{7,58} canine ^{59,60}	
Immobilization with compress	rabbit, ⁶¹ rat ⁶²	

cd = cell death
cl = cloning
eb = bone eburnation
nc = no changes
os = osteophyte
sb = subchondral bone
pl = proteoglycan loss
sf = surface fibrillation

specificity of antibodies or molecular biology probes. However, if the question is which model or models are best for the identification of disease modifying agents then the answer is extremely difficult. Naturally occurring arthritis occurs in many species including certain inbred strains of mice (C57bl, STR/1N, STR/ORT) and guinea pigs (Table 1). The difficulties with utilizing naturally occurring models are if they occur due to a genetic abnormality and the time of onset and severity.

Surgical models of mechanical instability which are mimics of chronic traumatic osteoarthritis are the most commonly utilized models. There are four prime advantages: incidence is 100%, time of onset is known, site where osteoarthritis develops is known and the duration can be short. But there are also disadvantages: variability in severity, size of animals (>20 kg dogs, 25 gm mice), age of animals (guinea pigs and mice immature while most dog ages are unknown), preexisting or naturally occurring osteoarthritis (guinea pig, dog, C57bl mice). The choice of animal again is

TABLE 3
Chemical Models with Joint Injection

Cell Perturbation	Species ^{Ref.}	Comments
Iodoacetic acid	rabbit, ⁶⁵ guinea pig, ⁶⁶ chicken, ⁶⁷ canine ⁶⁸	cd, sf, eb, cl, os, pl
IL-1b	rat ⁶⁹	pl
Vitamin A	rabbit ⁷⁰	erosion, os
Fibronectin fragments	rabbit ⁷¹	pl
H ₂ O ₂ and exercise	rabbit ⁷²	
Direct Matrix Depletion	Species	Comments
Trypsin	rabbit ⁷³	
Papain	rabbit, ⁷⁴ guinea pig ⁷⁵	cl, sf, pl
Chymopapain	rabbit ^{17,76}	pl, sl, eb, cl
Chondroitinase ABC	rabbit ⁷⁷	pl
Collagenase, bacterial	mouse ⁶³	
cd = cell death		
cl = clones		
eb = bone eburnation		
os = osteophyte		
sb = subchondral bone		
pl = proteoglycan loss		
sf = surface fibrillation		

TABLE 4
Osteoarthritis Model for Joints Other Than the Knee

Model	Animal ^{Ref.}	Comments
Lumbar facet joint (intradiscal chymopapain)	canine ⁷⁹	transient, early changes
Cervical facets-bipedal	rat ⁸⁰	
Hip — papain injection	rabbit ⁸¹	similar to knee
Hip — osteotomy	canine ⁸²	rapid aggressive change
Temporomandibular joint (natural)	mouse ⁸³	
Temporomandibular joint (surgical)	rabbit ⁸⁴	
Temporomandibular joint	rabbit ⁸⁵	
Wrist, rat, spontaneous	rat ¹⁸	

For keys see table 3.

dependent upon the question to be answered. Surgical models are available for use in mice,⁸⁶ guinea pigs,¹² rabbits,^{1,41} goats, sheep and dogs (Table 2). Mice are small, easy to handle and administer compounds, large numbers can be used, and transgenic animals can be studied; however, the anatomy of the joints differs significantly from that of humans in that mice do not go to full skeletal maturity (growth plates fail to close), the articular cartilage is only 3–5 cell layers thick (Figure 1), and the horns of menisci are ossified. Rabbits are more difficult to handle and to dose; the joint anatomy and gait (hop) differs significantly from that of humans. Goat and sheep are ruminants which are of concern when compounds are to be studied, are large (>50 kg), and stifle joint differs from humans anatomically in that it contains three patellar ligaments and synovial fossae develop.

Goats are also prone to develop caprine arthritis encephalitis, an inflammatory arthritis, which could complicate the study and analysis of joints.

Of all the surgical models, the canine anterior cruciate ligament transection model is the best characterized. The clinical, radiographic, morphologic, and biochemical changes that occur following surgery are similar to those found in naturally occurring osteoarthritis in dogs^{48,87} and humans.⁸⁸ The instability can be created either open through a medial^{38,89} or lateral⁸⁸ arthrotomy, or closed via a stab incision.^{38,87} However, the major limitation for analysis of pharmacologic agents is length of time (approximately 3–4 years),⁴⁶ that the dog takes to progress to clinical, not early, osteoarthritis.

E. GENERAL PRECAUTIONS

Good animal husbandry practices are important. The animal models, and especially the spontaneous models, may depend on the animal source and the cleanliness of the colony. If age is important, using animals of known birth date has advantages over weight, especially as they near maturity. If closed growth plates are required, X rays are recommended if the ages are unknown. Controlling stress levels is important and the environment (temperature, noise and lighting) and animal handling should be stress-free and controlled as much as possible. A controlled diet is also important since osteoarthritis progression is highly dependent on weight,⁹⁰ in the mouse,⁸ guinea pig,¹³ and dogs.⁹¹ Modest exercise or conversely deconditioning may have dramatic effects.¹⁹ This may be even more important as new larger runs are required for dogs and the animals may now be able to more frequently jump on their hind limbs and socialize. Enrichment for other animals may also change activity levels.

III. EVALUATION METHODS

A. CLINICAL EVALUATION

In vivo evaluation methods vary dramatically with the size of the animal. The methods can include physical exam, gait analysis, kinematics, arthroscopy, conventional radiography, magnetic resonance imaging (MRI), diagnostic ultrasound, computerized tomography (CT), microCT,³⁵ scintigraphy, and fluid analyses.

1. Physical Exam

A good physical exam is a necessity as part of the records. This should include comments on general health, weight, and temperature, noting food and water intake and hair and eye quality. The animals should be examined for altered gait, signs of joint swelling, redness of skin, temperature over the joint, active and passive range of motion of the control and experimental limb, joint stiffness, and the presence of instability. After surgery, wound healing should also be monitored.

2. Gait Analysis

Gait analysis using a strain gauge force plate can be done on animals as small as a mouse (single channel) to as large as a horse (6 channel). Determination of the ground reaction forces is a simple procedure which describes the forces and moments of the foot to ground contact that can be used as an indicator of limb (not joint) use under varying conditions. The forces can be described by orthogonal vector components including the vertical (F_z), craniocaudal (F_y ; braking and propulsion) and mediolateral (F_x). Gait analysis has been used in dogs to describe both the normal,³⁹ as well as that of dogs with either acute or chronic arthritis.^{46,89,92} While initial costs can be as high as \$30,000 to equip a gait analysis lab, the day to day costs are low. Equipment needs include a room, computer, force plate (AMTI or Kistler) and program.

3. Kinematics

Kinematics, the study of movements of the limb, has been used by investigators to study the normal gait and the changes that occur in dogs with anterior cruciate ligament deficiency,^{93–95} and/or massive hind limb deafferentation.⁹⁵ The change in kinematic parameters after transection of the anterior cruciate ligament was shown not to be limited to just the destabilized joint, but also affected the other joints in that limb, joints in the contralateral limb, and the vertical movements of the rump.⁹⁴

4. Arthroscopy

Arthroscopy can be utilized to visualize the surface of most components of the joint (articular cartilage, intra-articular ligaments, menisci and synovium), but it cannot be used to examine the deep structures (i.e., subchondral bone), joint effusion or extrasynovial structures. Furthermore, tactile inspection can also be made by probing and/or applying compression. Currently, due to the size of the scopes, arthroscopy is limited to larger animals. It is invasive which can have effects on the joint, can be painful thus impacting on the model if the animal does not use the limb, requires anesthesia and thus post-procedure care, significant operator training is necessary, and it is expensive.

5. Radiography

Imaging techniques have also been used to follow the development of arthritis. Conventional radiographs allows 2D evaluation of secondary changes of osteoarthritis, however, joint space (cartilage space) cannot be addressed unless views are made during weight-bearing which is difficult to impossible to accomplish in most animals. Radiographic grading schemes using standard views of synovial joints have been developed and utilized to describe the soft and hard tissue changes in both experimental and naturally-occurring osteoarthritis in most species.⁹⁶ In dogs, Widmer *et al.*⁹⁶ were able to describe the development of osteophytes as early as two weeks following anterior cruciate ligament transection in dogs. They attributed the early identification to their use of five different views. Arthrography can be used to delineate intra-articular surfaces of cartilage, menisci, ligaments and synovium. If the equipment is present on site, radiographs are inexpensive and easy to take; however, analysis can be difficult and requires training and rigorous standardization.

6. MRI

Magnetic resonance imaging has been used to describe the progression of osteoarthritis in mice,¹¹ rat,⁹⁷ guinea pigs,⁹⁸ rabbits,⁹⁹ and dogs.^{46,96} MRI provides exceptional soft tissue contrast not afforded by other imaging methods. Therefore, internal components of synovial joints, i.e., menisci, synovium, ligaments, etc. can be seen. While MRI can be useful in following the development of osteoarthritis over time, it is limited in the size of animal that can be examined, the expense of the equipment and the training necessary to evaluate the images. MRI of small animals requires the availability of instruments with strong magnetic fields in order to obtain good resolution.^{11,97,98}

7. Ultrasound

Diagnostic ultrasound also allows evaluation of internal components of synovial joints, but is limited to medium to large joints and is extremely operator dependent. The normal ultrasonic appearance of the canine stifle joint has been described,⁴⁷ however, the ultrasonic appearance of the arthritic joint has not been previously described.

8. Scintigraphy

Scintigraphy with bone seeking radiopharmaceuticals provides excellent physiologic and limited anatomic information regarding bone turnover and early soft tissue changes associated with osteoarthritis. These radiopharmaceuticals are used to study both hard and soft tissue changes associated with osteoarthritis. Bone phase scintigraphic evaluation is sensitive for identifying initiation of osteophytosis and subchondral remodeling. Blood pooling and soft tissue phase imaging will detect the early inflammatory changes of synovitis. In rabbits the radiopharmaceutical ^{99m}Tc methylene diphosphonate was increased in operated knee joints in the area of developing osteophytes as early as one week post destabilization.¹⁰⁰ Later uptake occurred in the subchondral bone beneath areas of damaged cartilage. Tc-labeled macrophage methodology is also used as a way of detecting low levels of synovitis in rabbit osteoarthritis models.¹⁰¹

9. Fluid Analyses

Serum, urine, and synovial fluid can be evaluated biochemically for its content of cartilage breakdown products (i.e., keratan sulfate)^{102,103} and/or production of mediators (i.e., TNF, IL-1, matrix metalloproteinases) as well as parent compound and metabolite levels.¹⁰⁴

B. MACROSCOPIC ANALYSIS

Following euthanasia, the joints of interest should be inspected for changes in muscle mass, thickening of the joint capsule (specific anatomical areas noted), and the synovial fluid collected (color, turbidity, and amount noted) and centrifuged to remove cells, aliquoted and stored at -80°C . If inflammation is suspected, cytopspins of the cells can be made, and cell counts and cell identification performed. The joint should be opened and the synovial membrane (all aspects), ligaments, articular cartilage (extent and depth), bone changes (osteophytes, eburnation, etc.) and if present menisci (striations and or tears) all inspected and scored.¹⁰⁵ To highlight early changes to the articular cartilage India ink can be used.⁶⁴ Menisci can be scored for texture (smooth, linear streaking, irregularities, or tears), thickness (normal, thinned and translucent, or enlarged inner circumference) or tears (location, size, number).¹⁰⁵ Samples from synovium, cartilage and bone should be taken either into a fixative or frozen for further analysis. Photographs taken at necropsy can provide useful records for review if needed.

C. MICROSCOPIC ANALYSIS

In setting up samples for evaluation for the first time with general histological, immunohistochemical or molecular biology protocols, specific methods and references should be consulted.¹⁰⁶ There are a few alterations that may be needed because of the unusual nature of cartilage with its low cellularity and presence of high concentrations of polyanionic proteoglycans. The presence of bone further complicates the situation with slow fixation, and the added need for decalcification.¹⁰⁷

Troyer⁷ suggests that preparation of samples for general histology by paraffin embedding of decalcified tissue should include fixation with a buffered fixative, such as formaldehyde, and with the smallest size sample that is practical. Decalcification in citrate and EDTA in the cold is the gentlest method, but time consuming.⁷ For many purposes, 10% formic acid or 10% formic acid/3.7% formaldehyde at room temperature has given acceptable results.³¹ For samples embedded in paraffin to prevent specimen hardening and being more difficult to cut, the paraffin temperature should be kept only a few degrees above the melting temperature and chloroform should replace xylene in the dehydration series. Glycol methacrylate resins offer an attractive alternative mounting media.⁷

Samples for immunohistochemistry may have to be fresh-frozen and a frozen section cut if the antisera only detects the native protein. In some cases, renaturation of fixed tissue in a microwave may be possible. For good antisera penetration and epitope unmasking, proteoglycans frequently must be removed by digestion with hyaluronidase or chondroitinase ABC or AC.¹⁰⁸

Most investigators use histopathology as an end point in their studies. However, one major limitation is an adequate validated grading scheme. Many grading schemes have been developed and used to describe the changes that occur in the articular cartilage observed in osteoarthritis,^{38,108,109} and some have even been semi-automated.¹¹⁰ The most widely adopted grading method for evaluating microscopically hyaline cartilage changes was first described by Mankin et al. in 1971.¹⁰⁹ However, the Mankin scheme has been described by one group to be reproducible,¹¹¹ and by others to be inadequate.^{38,112} Furthermore, the use of the histochemical stain Safranin O has also been questioned by some researchers for its lack of reproducibility.¹¹³ Thus the need for a reproducible, standardized and validated grading scheme still exists and may involve scoring of area and severity of the surface fibrillation. For small animals, if no other analyses are planned, it may be possible to do this by scanning electron microscopy.^{14,19}

Grading schemes have also been developed to assess the changes that occur in the synovial membrane.^{38,114} In general the amount of mononuclear cell infiltrate, deposition of hemosiderin and recently the presence of formalin-resistant mast cells have been evaluated.³⁸

Cartilage preparation for transmission electron microscopy (TEM) with good cell and matrix preservation requires additional care. The best example of cartilage preservation has been with high pressure, low temperature freeze substitution methods,¹¹⁵ but this requires expensive equipment and is technically demanding. Routine fixation for TEM is usually done with a ruthenium salt present in the fixative if proteoglycan preservation is required, although this may slightly compromise the cell membrane details.¹¹⁶ Scanning electron microscopy for surface detail requires careful regulation processing to prevent cracking artifacts.^{14,19,117}

D. MOLECULAR BIOLOGY METHODOLOGY

In situ hybridization offers many of the advantages of immunolocalization since the cellular response in the different regions of cartilage can be easily determined and even quantitated.¹¹⁸ The major advantage of *in situ* hybridization over immunohistochemistry is that by looking at message RNA, short-term cellular responses can be evaluated. The major caveat being that not all mRNA expression leads to protein production. For low abundance messages, the signal can be further enhanced using *in situ* RT-PCR.¹¹⁹

Extraction and purification of mRNA for cartilage and other dense connective tissues requires extra care to release the mRNA from the relatively acellular tissue and remove the contaminating proteoglycans and can be used on as little as 10 mg of tissue.^{120,121} However, in the small animals, even this small amount of tissue represents pooled samples and may still include involved and noninvolved tissue. Although direct analysis of mRNA levels is something possible,¹²² RT-PCR has been frequently employed in both semi-quantitative and quantitative competitive RT-PCR applications.^{123,124}

E. BIOMECHANICAL PROPERTIES

Biochemical components and structure of articular cartilage and subchondral bone determine their mechanical properties. Intrinsic (such as age effect) or external (impulsive loading or fracture) factors cause biochemical and morphological changes of articular cartilage and subchondral bone. Then, mechanical properties of these tissues follow. Biomechanical properties can provide important information about the process, especially early in the process before gross histologic changes occur. These very specialized measurements can extract intrinsic material properties of juxtaarticular bone.^{58,60,125} See Chapter 9 and 16 for methods of cartilage mechanical testing.

IV. APPLICATIONS OF THE MODELS

A. EVALUATION OF THE PATHOPHYSIOLOGY OF OSTEOARTHRITIS

Early-stage human samples are difficult to identify or obtain. The animal models have been used extensively for defining early and mid-stage disease and providing comparisons between disease progression in different species and different models. Because of the larger amounts of tissue available, most of the biochemical and biomechanical studies have largely focused on the canine, rabbit and sheep models.^{13,72-74} Extensive histologic studies and some immunohistochemical studies have been done on all the models. Molecular biology methods, especially RT-PCR^{122,124} and *in situ* hybridization methods have seen limited,¹¹⁸ but expanding use.

The important questions currently being addressed with animal models are: what is the nature of early stages of the disease, what causes early matrix swelling and the prolonged hypertrophic phase, what are the enzymes involved in matrix turnover and what is their relative contribution to disease progression, how does initial trauma lead to subsequent degeneration, how much of an active role do chondrocytes play in progression, how do joint biomechanics contribute to disease progression, what role do other tissues, especially bone, play in osteoarthritis, what is the interplay of genetics, environment and mechanics in osteoarthritis, and at what stage is the process still reversible?

B. INTERVENTION AND OSTEOARTHRITIS MODELS

The frequency of osteoarthritis models used in interventions appears to be strongly driven by cost, ease of animal handling, especially of intra-articular injections, rapidity of disease development, and to a lesser extent, availability of the appropriate reagent. This means that, to date, rabbit models were used several times more often than the canine ACL model with a smattering of other species. Most of these interventions have been aimed at slowing early stages of progression with group sizes of 4–20 animals for 4–12 weeks, and either gross or histologic measures have been used to assess the extent of cartilage destruction, which are usually measured by fibrillation and loss of cartilage height as the read-outs. The majority of intervention studies have only been able to test a single level of compound and/or dosing, a single dosing schedule, so previous *in vitro* proof of efficacy and possible effective levels of drug are important. Table 5 compares the response of humans with osteoarthritic cartilage and animals to various drug treatments or pharmacologic interventions.

C. IDENTIFICATION OF MARKERS OF OSTEOARTHRITIS

The investigators in the field have an intense interest in identifying molecular markers that could be used to identify patients at risk, to follow disease progression, and be used to demonstrate efficacy of interventions. The possible complications of following development of the disease in a single joint by assaying urine, blood or synovial fluid have been extensively discussed.^{103,146} Some of the problems include the signal to noise ratio from normal metabolism by the noninvolved joints or other tissues, the specificity of the marker for the tissue and disease process, or altered marker turnover either by changes in metabolism or flux (especially in synovial fluid) caused by disease.^{147,148} Synovial fluid has the advantage of being directly from the involved joint. The major drawbacks are in obtaining enough sample volume and the relationship of the fluid levels to actual tissue levels and turnover in the tissue. Serum levels can reflect the summation of all possible sources, while the urine products can also be influenced by metabolism in the kidney.

Since animal models can be followed from the time of initiation of the process, models are being used to determine if and how a given marker changes with disease progression and to follow up biochemical leads from culture experiments. Samples from human osteoarthritic patients are being used to follow mid- and end-stage disease. The field has focused on cartilage-specific or cartilage-enriched markers, which change in the osteoarthritic process. There is renewed interest

TABLE 5
Comparison of Human Osteoarthritis with Animal Models
of Osteoarthritis — Pharmacology and Therapeutics

	Human ^{Ref.}	Dog ^{Ref.}	Rabbit ^{Ref.}	Guinea Pig ^{Ref.}	Mouse ^{Ref.}
Modulation of Inflammation/pain					
Corticosteroids	↓ ¹²⁶	↓ ¹²⁷			
NSAIDS	↓ ^{128,129}				
Tenidap					
Hyaluronic Acid	↓ ¹³⁰				
Polysulfated GAGs	↓ ¹²⁹				
MMP Inhibitors					
Doxycycline					
Modulation of Chondrodestruction/osteophytosis					
Corticosteroids	↑ ¹³¹	↓ ¹³²	↓ ⁴⁰	↓ ¹³³	No ¹³⁴
NSAIDS	No/↑ ¹²⁷	No ¹³²	No/↓ ¹³⁵		No /↓ ¹³⁵
Tenidap	? ¹³⁶	↓ ¹³⁷			↓ ¹³⁸
Hyaluronic Acid		↓ ^{139,140}			
Polysulfated GAGs	↓ ¹²⁹	↓ ¹²⁷	↓ ¹²⁷		No/↓ ¹²⁷
MMP Inhibitors			↓ ¹⁴¹	↓ ¹⁴²	
Doxycycline		No ↓ ¹⁴³	↓ ¹⁴⁴	↓ ¹⁴⁵	
NSAIDS = nonsteroidal anti-inflammatory drugs					
GAG = glycosaminoglycan					
MMP = matrix metalloproteinase					

in finding fragments of matrix molecules that could be used either as markers of catabolism or anabolism, or tissue remodeling.¹⁰⁴

In animal models, a few examples include: the persistent upregulation of serum hyaluronic acid,¹⁴⁹ change in keratan sulfate levels,^{102,103} specific sulfation epitopes in chondroitin sulfate,¹⁵⁰ specific degradation products of aggrecan, bone sialoprotein (a marker for bone remodeling), metalloproteinases, cartilage oligomeric matrix protein, and type II collagen fragments including amino and carboxyl crosslink fragment(s) and link protein fragments.¹⁰⁴

D. BONE INGROWTH TO IMPLANTS UNDER OSTEOARTHRITIC CONDITIONS

Since there are many studies that demonstrate changes of the subchondral bone in osteoarthritis,^{107,151} it is possible that as implants are developed that allow replacement of only part of the osteoarthritic joint, there may be problems with bone ingrowth. This has been reported for rheumatoid arthritis, diabetes, or revised joint replacements.¹⁰⁷ End-stage, large animal models of osteoarthritis would be useful for these studies. Ideally, the model would be allowed to develop to at least mid-stage where bone changes are extensive and then the mechanical defect corrected by surgery before the implant study is started.

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19 Animal Models of Rheumatoid Arthritis

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I. INTRODUCTION

Animal models of rheumatoid arthritis have been sought and employed in research for decades, but, as in other complex human conditions, no animal model is identical to the human disease. The disease states which are created as animal models of rheumatoid arthritis are thus inflammatory arthritides but cannot be considered to be “rheumatoid” arthritis.

Nevertheless, these models have afforded many opportunities to better understand the pathology, etiology and pathogenesis of rheumatoid arthritis (RA), as well as genetic factors in the disease. There is a growing number of reports on the use of animal models for experiments relevant to the surgical management of RA. Until the appearance of new methods of genetic manipulation permitting the development of animal strains which more closely mimic the natural course of disease, no specific species of animal was identified which naturally developed inflammatory arthritis similar to clinically observed human rheumatoid arthritis.

The earliest studies seeking an appropriate model of rheumatoid arthritis in the animal were conducted in the monkey, horse, sheep, goat, pig, dog rabbit, guinea pig, rat, mouse and chick embryo.¹ Arthritis was induced by injection of various antigens, either directly into the joint, or into subcutaneous or serous tissues for systemic effect. Many antigens have been injected in an effort to create inflammatory joint disease, and the arthritogenicity of various antigens was determined on an empirical basis.¹ The nomenclature for models of rheumatoid arthritis continues to reflect the specific antigen used to induce arthritis. Models of arthritis are sometimes categorized into monoarthritic and polyarthritic types. All animal models of arthritis are based on the injection of an irritant or antigen which results in an inflammatory or immunological response which is either local or generalized. Although sharing this common factor and often a similar general pathology, each model is characterized by a particular pathogenesis, course of disease, and pattern of joint and extraarticular involvement. The current rapid progress in understanding the genetics, cell biology, and cytokine chemistry of various models is increasing our fundamental knowledge of models which have, in some cases, been in use for decades.

The use of several different types of animal models, with known differences in etiology and pathogenesis, permits differential approaches to an understanding of mechanisms of arthritis. As interventions or treatment modalities are introduced into the model, hypotheses regarding the expected mechanism of action or pathological process which is affected can be tested against one or more of the models.² A considerable variety of animal models of rheumatic disease have been developed reflecting the wide range of signs and symptoms which characterize the various human diseases. Animal systems developed to model the spondyloarthropathies, systemic lupus erythematosus, systemic sclerosis, Sjögren's syndrome, polymyositis, and dermatomyositis have been comprehensively reviewed.³ Models of inflammatory joint disease which are induced by viable infectious agents are of particular interest in the study of the “trigger” event which is presumed to initiate a pathological immune response in those individuals who are genetically susceptible to the development of autoimmune diseases such as rheumatoid arthritis. Mycoplasmas may be the most common cause of naturally occurring arthritis.

TABLE 1
Animals Employed for Models of Rheumatoid Arthritis

Animal	AA	CIA	SCW	AIA	CarA	Ref.
Mouse	DA	DBA/1		CBA		3, 5, 50
Rat	Wistar	Wistar, SD	SD, Lewis			8, 9, 10, 24, 39, 40
Rabbit				NZW, Old English	NZW	5, 42, 44, 68
Dog					mongrel	78
Pig					SPF	49
Monkey		squirrel, macaque				50

AA = adjuvant arthritis

CIA = collagen induced arthritis

SCW = streptococcal cell wall induced arthritis

AIA = antigen induced arthritis

CarA = carrageenan induced arthritis

SPF = specific pathogen free

Rheumatoid arthritis creates a specific spectrum of musculoskeletal pathology, different in important ways from other categories of disease, such as osteoarthritis. Rheumatoid involvement of articular surfaces, ligaments and joint capsules, juxtaarticular and diaphyseal bone, and tendons and tendon sheaths creates characteristic symptoms and impairments requiring specific orthopaedic management. For this reason, an evaluation of the response of a bone, joint or soft tissue structure in rheumatoid disease to a manipulation or surgical intervention should be evaluated not in a normal animal, nor in a model of arthrosis, but, when possible, in an analogous animal model of inflammatory arthritis. The focus of this chapter has been placed upon those animal models of rheumatoid arthritis which are most relevant for experiments on the surgical management of the musculoskeletal system, and upon practical applications of these methods to surgical questions. A narrow definition of the scope of orthopaedic research has been avoided, and studies on implant fixation to abnormal bone, synovectomy, bone anatomy and biomechanics are reviewed, as well as studies of the mechanism of joint destruction in inflammatory disease. A detailed review of the genetics and biochemistry of models of inflammatory arthritis is beyond the scope of this chapter: Excellent reviews are available.⁴⁻⁶

II. SELECTION OF AN ANIMAL MODEL OF RHEUMATOID ARTHRITIS

Table 1 lists the most commonly used animal models and major references which provide detailed information on animal models of rheumatoid arthritis (RA). The murine species, the rat and the mouse, are the most commonly used because they are inexpensive and easy to handle and breed. The use of canine and porcine models of arthritis, and to a lesser degree lapine models, is limited by the cost of handling and maintenance of the animals. It is widely considered that results obtained in experiments in large, long-lived mammals are most relevant to human disease, especially where the anatomy or physiology of the smaller animal is known to differ from the human. One example of an important species-related difference is that the skeletons of small rodents do not mature, but continue to grow throughout life. This growth pattern is termed a modeling skeletal system, which differs from the pattern in humans and other large mammals, in which the skeleton is considered to be a remodeling system after the cessation of longitudinal growth. Notwithstanding this principle, where larger species have been employed in studies of arthritis, the findings have proved in general to be comparable to those for either the rat or the mouse. It is advisable to consider the specific

anatomical or physiological factors of importance to the specific experiments to be carried out in order to determine if the proposed species is an appropriate model. In some cases, the use of larger species, particularly the rabbit or the dog, is based on a large existing literature describing the histology, pathology or physiology of the animal and its relevance to human disease. In studies in which biomechanical testing is performed, or where a surgical intervention or implantation is planned, larger specimens are usually employed because of the requirements of larger samples.

A. ADJUVANT ARTHRITIS

Adjuvant arthritis was introduced and developed by Pearson⁷ and more extensively studied by Pearson,⁸ Taurog,⁹ van Eden, their coworkers and many others. The original and more recent literature on the topic of adjuvant arthritis is extensive, and the interested reader is directed to several reviews.^{6,10–12} Early experiments studied the effect of various chemical irritants and antigens in inducing arthritis; ultimately a water-in-oil adjuvant was found to be effective.⁷ Adjuvants are water-in-oil emulsions or simple mixtures in oil, which are more effective in inducing arthritis than are oil-in-water emulsions. Freund's adjuvant is termed "complete" when it contains killed mycobacteria of any of several species.

1. Induction of Arthritis

Adjuvant arthritis is induced in either the laboratory mouse or in the rat. The success of induction of arthritis is species-dependent and also strain-dependent.¹³ In-depth reviews of the types of adjuvants and their use are given by Whitehouse et al.,¹⁴ Herbert,¹⁵ Whitehouse,¹⁶ and Taurog.⁶ In more recent research, the most commonly used adjuvant material is a complete Freund's adjuvant, prepared without water. Lyophilized *M. tuberculosis* or *M. butyricum* is prepared as a suspension in purified mineral oil or paraffin oil using a tissue grinder, and can be stored at 4°C for a period of up to six months. In commercially available complete Freund's adjuvant the concentration of active antigenic material may be lower than the required 0.5–1.0 mg per dose. Immediately before intradermal injection at the preferred site at the base of the tail, the material is mixed again using a vortex.

2. Clinical Observations

Depending on the species, size and strain of animal, the success of intradermal injection (providing a depot for slow release of the antigen), antigenic preparation, vehicle, and concentration and dose of the adjuvant employed, arthritis will become evident in the hind limbs within 11–21 days after the injection. Elevated temperature, redness and swelling of the hind feet, and occasional paresis of the hind limbs may also be observed. Scoring systems have been developed for the grading of these clinical signs. Necrosis may appear at the site of injection and extend distally along the length of the tail.

3. Gross Pathology and Histology

Histological examination of the affected hindpaw joints shows an acute synovitis, joint effusion and proliferation of synovium, usually without a purulent effusion; a subsynovial infiltration consisting of histiocytes, lymphocytes and plasma cells with a few polymorphonuclear leukocytes; peritendonitis and bursitis; invasion of subchondral bone by pannus; and new bone formation near the affected joints.

Further changes which may be observed from days 20–300 after the onset of arthritis include: fibrous thickening of the joint capsule, fibrous adhesions between articular surfaces, hypertrophy of synovial villi, a low grade inflammatory reaction with lymphocytic infiltrates near some joints, and periarticular ossification with occasional bone ankylosis, especially in the tail vertebrae.

TABLE 2
Comparison of Selected Animal Models of Arthritis to Rheumatoid Arthritis*

	RA**	AA	CIA	SCW	AIA	CarA
Onset/Course		100%	40–60%	100%	90%	100%
acute		10–12 days	14–60 days	3 days	21 days	28–49 days
chronic				14 days	40–300 days	
flare/remission	+			+	inducible flare	
Joints Affected						
peripheral	+	hind feet	hind feet	feet	tibio-femoral	tibio-femoral
axial		tail				
Extra-Articular Features	+	+	+			—
Immunity						
rheumatoid factor	+				+	—
collagen Type II ab	low	—	+	—		—
antibody response	+	—	+ / —			—
T cell response	+	+	+	+		—
complex formation	+				+	—
Soft Tissues						
cytokines in joint space	+	+		+		?
pannus	+	+	+	+	+	+
periosteal reaction	—	—	—	—		+
Effective Therapies						
NSAIDS	+	+	+ / —	+	—	
corticosteroids	+	+	+	+	+	
methotrexate	+	+	+	+	+	
gold salts	+	+ / —	worsens	—		
penicillamine	+	—	+	—		
cytokine antagonist /antibody			+	+	+ / —	
bisphosphonate		+			+ / —	?

* Table adapted and modified from Reference 50.

** RA = rheumatoid arthritis; other acronyms are the same as in Table 1.

Microscopic examination of the regional lymph nodes and other tissues indicates that the inoculum is transported by the lymphatic system to the circulatory system soon after injection, reaching the lung and liver where a classic granulomatous response occurs. Table 2 shows a clinical and pathologic comparison of adjuvant disease to other models and to rheumatoid arthritis, while Table 3 outlines bony effects.

4. Immunology

The immunological nature of adjuvant arthritis was recognized early by Waksman and Wennersten, who achieved the induction of adjuvant arthritis in rats by the passive transfer of living lymphoid cells from sensitized donors to their normal counterparts. The Lewis strain, which is most commonly used in adjuvant arthritis experiments^{13,17} and Wistar strains are particularly susceptible to adjuvant arthritis, while Fischer and BN rats are resistant.

B. COLLAGEN INDUCED ARTHRITIS

Since type II collagen is present only in hyaline cartilage at birth, an immune reaction to type II collagen generates a specific reaction in synovial joints. Antibodies to type II collagen are present

TABLE 3
Reported Effects on Bone in Selected Animal Models of Arthritis —
Comparison to Rheumatoid Arthritis*

	RA**	AA	CIA	SCW	AIA	CarA
Radiography						
radiographic lucency	+	+	+	+	+	+
joint space narrowing	+	+	+	+	+	
erosion	+	+	+	+	+	+
bone mineral density	+	+				
Joint						
joint stiffness	+					
tenosynovitis	+				+	
osteophyte formation	+	+	+	+	+	
ankylosis		+	+	fibrous		
instability / dislocation	+					+
Microscopic effects						
mineralization						+
osteopenia	+	+				+
structure						+
bone formation	variable					+
bone resorption	+					+
Mechanical properties	+					+
Bone marrow effects						
bone marrow cells		+	+			+
cytokines of bone	+	+	+			?
Therapies that alter hard tissue response						
NSAIDS					+	
corticosteroids	+	+			+	
methotrexate					+	
cytokine antagonist/antibody				+		
bisphosphonates						+
Altered osseointegration					no effect	

* Table adapted and modified from Reference 50.

** Acronyms are as shown in Tables 1 and 2.

in the synovial fluid of rheumatoid arthritis patients.^{18,19} However, antibodies to denatured collagens are also present in RA and in several unrelated diseases, suggesting that antibodies to collagen represent a secondary phenomenon in chronic inflammatory arthritis and are not reflective of the etiology of the disease.

1. Induction of Arthritis

In 1977, Trentham and colleagues recorded that 40% of rats injected intradermally with native type II collagen obtained from human, chick or rat cartilage developed an inflammatory arthritis.²⁰ Collagen prepared at a concentration of 1 mg/ml in 0.1M acetic acid was emulsified with an equal volume of complete or incomplete Freund's adjuvant; 1 ml of this preparation was injected in the back intradermally in four to six sites in the rat. After 21 days, a booster dose consisting of another 0.5 mg collagen was injected intraperitoneally without adjuvant. Other injection schedules utilizing type II collagen from human, chick or rat cartilage sources and complete or incomplete Freund's adjuvant, but not types I or III, nor the α chains of type II collagen, also are effective.²⁰

2. Clinical Observations

In the animals which developed arthritis, the onset occurred acutely at 20 days and persisted for as long as 60 days after immunization. On the first day of arthritis, the arthritic index in one hind limb typically was 4, the highest score. In some cases, both hind limbs were involved.

3. Gross Pathology and Histology

Histologic sections of the joints collected within 24 hours of the onset of arthritis demonstrated a mononuclear synovial infiltrate chiefly composed of T helper cells with few cytotoxic T-cells rather than a neutrophilic infiltrate.^{21,22} In the synovial fluid most cells were granulocytes with fewer macrophages, comparable to RA.²² The synovium and serum contained similar amounts of type II collagen antibody and rheumatoid factor.²² Samples collected at later stages show proliferation of synovium and fibroblasts, and erosion of cartilage and subchondral bone. Periosteal new bone formation progresses in some cases to joint ankylosis in the carpal, tarsal, metacarpal, metatarsal and interphalangeal regions. Presence of mononuclear cells was observed in the synovium for as long as six months after disease onset. There was no axial skeletal involvement. The incidence of arthritis following injections of Type II collagen did not vary with injections ranging from 0.5 to 2 mg per animal.

Chronic collagen induced arthritis of four to eight months duration resulted in bony ankylosis after total destruction of cartilage and new bone formation.

4. Gender Effects

In humans, the increased risk for females (3×) of developing rheumatoid arthritis has been speculated to be related to sex hormones and to the central nervous system. In collagen induced arthritis, gender differences are opposite in effect for the rat and the mouse.²³

5. Immunology

Collagen induced arthritis can be transferred passively from one animal to another by activated lymph cells. Nine of 32 naïve rats developed arthritis after receiving pooled spleen and lymph node cells from donors previously injected with type II collagen. Arthritis was not induced by injection of Freund's adjuvant alone, sera from arthritic donors, cells from nonimmunized donors, cells from rat donors which had been injected with type I collagen, cells from nonimmunized donors with solubilized type II collagen, or heat-killed cells from immunized donors. These controls established that disease transfer was not due to transfer of antigen, or chemical components in sera, but rather to transfer of sensitized cells.²⁴

The significance of the complement system in collagen induced arthritis is shown by three types of evidence: Affected mice produce high levels of antibodies of the isotypes which are most efficient at activating complement, and increases in C3 correspond with the development of antibody. Administration of cobra venom which depletes complement prevents development of disease. Passive transfer of the disease is successful only in animals which have normal levels of complement.

T-cells have an essential role in collagen induced arthritis. Passive transfer of the complete disease is achieved only when T-cells are involved. Administration of anti-MHC Class II antibodies or anti-CD4 antibodies attenuates the disease, and arthritis cannot be induced in athymic nude rats which lack T-cells.^{25,26} Pretreatment with antibodies to the T-cell receptor also significantly reduces the incidence of arthritis.

Comparison of the immunogenetics of RA and collagen induced arthritis is relevant because of the importance of MHC Class II genes in the human disease. Experiments describing the role of the comparable genes in mice in collagen induced arthritis are reviewed by Crofford and Wilder.³

A genetic factor was also identified in rats: the dr bb/WOR-UTM strain developed collagen induced arthritis with an incidence of 100%.

Animals injected with other types of collagen raise an immune response which is not arthritogenic. The injection of heterologous type II collagen results in antibody development which is specific to particular epitopes of the molecule. The region which contains these epitopes has been dubbed CB11 as it is contained within the cyanogen bromide fragment.²⁷ The immune response in collagen induced arthritis does not rely on the production of a single antibody for a particular epitope, but rather a combination of antibodies, which together produce the full arthritis response.²⁸ Persistent cases of collagen induced arthritis demonstrate interaction of humoral and cellular aspects of immunity.²³ The active induction of arthritis is prevented by the application of specific monoclonal antibodies to T-cells²⁵ and the passive immunization model of collagen induced arthritis is prevented by depletion of inflammatory fibrocytes.²⁹

The concept of oral tolerance in RA and other inflammatory arthritides was developed through studies of collagen induced arthritis and adjuvant arthritis. The role of the T-cell receptor and the interaction of T-cells and B-cells as exemplified by interactions of the CD40 ligand GP39 present on activated CD4 T-cells is reviewed in detail by Durie et al.²³

6. Significance of the Collagen Induced Arthritis Model

This model was of contemporary importance in demonstrating that homologous tissue could be arthritogenic, independent of complete Freund's adjuvant which contains bacterial cell walls. Features of collagen induced arthritis comparable to those of rheumatoid arthritis include proliferative synovitis in a chronic, polyarticular, erosive disease exhibiting symmetrical involvement of small and medium-sized peripheral joints, sparing of the axial skeleton, erosion of cartilage and subchondral bone at joint margins, and immunity to native Type II collagen³⁰ (Table 2).

Collagen induced arthritis is distinct from other models of arthritis in that it is induced by an endogenous antigen, and also because arthritis can be induced in naïve animals by the injection of anticollagen antibodies. As has been observed and reported for RA, the cytokines interleukin 1 (IL-1) and tumor necrosis factor (TNF- α) appear to be involved in the pathogenesis of collagen induced arthritis (reviewed by Durie et al.²³). For example, recent studies employing the IL-1 receptor antagonist (IL-1ra), show that edema, cellular infiltration, and increased proteoglycan levels in synovial fluid during the induction phase of adjuvant arthritis are not inhibited, but IL-1ra has an anti-fibrotic action, decreasing abnormal interstitial collagen deposits and promoting regeneration of normal fat spaces in the synovial lining.^{31,32} Collagen induced arthritis differs from RA in that subcutaneous nodules and pulmonary fibrosis are lacking, as are extraarticular manifestations, with the exception of lesions in the hyaline cartilage of the ear.

Adjuvant arthritis and collagen induced arthritis are similar in exhibiting immunologic hypersensitivity to type II collagen. Sera from rats with adjuvant arthritis contains increased amounts of hemagglutinating antibodies specific for type II collagen, and also for the α chain.³³ Also, peripheral blood mononuclear cells collected from rats with adjuvant arthritis respond to homologous type I and also type II collagen by increased incorporation of tritiated thymidine.²⁰ Trentham and colleagues proposed that adjuvant activity and arthritogenicity are properties derived from different portions of the glycopeptides contained in mycobacterial cell walls. Trentham's group also demonstrated that intact telopeptide regions of the type II molecule are not required, since pepsin-modified molecules were arthritogenic. A significant drawback of collagen induced arthritis is the technically complex preparation of the inducing agent which may become contaminated by other bacterial products, particularly proteoglycans, which have arthritogenic and immunomodulating properties.³⁴

Another drawback of collagen induced arthritis is the variable onset from day 20 to 48 after the initial injection.³⁰

C. PROTEOGLYCAN INDUCED ARTHRITIS

Proteoglycan can be extracted from cartilage matrix digestion with chondroitinase-ABC and injected with Freund's complete adjuvant to induce arthritis in a susceptible mouse strain, BALB-c. Nine to twelve days after the second booster injection at four weeks, 100% of injected animals develop arthritis which becomes severe 7–8 weeks later.³⁴ Chronic disease involving cartilage and bone destruction, osteophyte formation and ankylosis, including ankylosis of the spine follows. Both humeral and cell-mediated immunity are involved as demonstrated in experiments with successful transfer of disease by injection of sensitized B- and T-lymphocytes into naïve recipients.

D. ANTIGEN INDUCED ARTHRITIS

The term “antigen induced arthritis” includes a heterogeneous group of conditions induced by various antigens and methods. The most commonly used antigens are methylated bovine serum albumin and ovalbumin. Early models of antigen induced arthritis were described in the rabbit by Dumonde and Glynn³⁵ and the mouse³⁶ and, more recently, the rat. A thorough review which emphasizes lapine antigen induced arthritis and contains many practical details and instructive illustrations was published by Cooke.⁵

Induction of rabbit antigen induced arthritis begins with intradermal or subcutaneous injection of 5 mg BSA or ovalbumin in 1 ml of emulsion at multiple sites in the back. Original protocols followed with one or two booster injections, after 14–21 days, which have been omitted by some recent investigators.³⁷ Sensitization is confirmed by a positive skin test at 21 days, demonstrating presence of a delayed type hypersensitivity response prior to the arthritogenic intraarticular knee injection.^{32,37,38} The success of induction varies with species; the rabbit is hyperresponsive so that anaphylactic shock may occur, while induction in the mouse, and particularly, the dog, is much more difficult.⁵

Within a few hours of injection, monoarticular arthritis begins with acute synovitis, swelling and exudate. Over two weeks, this is replaced by the chronic phase, characterized by invasive pannus and cartilage erosion, which persists as long as 24 weeks.³

E. STREPTOCOCCAL CELL WALL ARTHRITIS

Models of arthritis which employ an arthritogen developed from bacterial components are relevant to arthritis associated with Reiter's syndrome, inflammatory and infectious bowel diseases, rheumatic fever, post-Streptococcal arthritis, and Lyme arthritis all of which appear to be associated with bacterial infection. Although arthritis is inducible in animals by injection of bacterial components from a number of species: *Lactobacillus casei*, *Eubacterium aerofaciens*, *Bifidobacterium* species, and *Peptostreptococcus productus*, the most commonly used agent is *Streptococcus pyogenes*.³ Streptococcal cell wall arthritis was developed in the 1950s and 1960s from the observation that injection of Streptococcal cell wall material including a peptidoglycan polysaccharide complex which resists biodegradation and persists in tissue, created an inflammatory lesion in the skin of the rabbit.³⁹

Arthritis was first induced in rabbits by intraarticular injection and in the rat by intraperitoneal injection. In mice, a similar protocol results in pericarditis rather than arthritis. A whole cell sonicate is prepared from cultures of viable cells by treating them with 90 minutes of ultrasonic vibration, followed by filtration. The rhamnose content of the material is measured and adjusted in order to deliver 60 mcg rh/g body weight intraperitoneally. Alternatively, a heat inactivated whole cell preparation is prepared in phosphate buffered saline by adjusting the turbidity to compare with a previously standardized whole cell sonicate. About 10% of animals develop a mild arthritis which disappears within four days. Ninety percent of animals later develop one of two distinctive forms

of disease. In one pattern animals have a period of complete remission followed by severe recurrence after 60 days. In the other pattern, arthritis subsides and then recurs several times over the study period of 130 days, without complete remission. The onset of disease is rapid, within 15–48 hours, marked by the appearance of red, swollen tarsal, carpal and interphalangeal joints.

1. Pathology

The heart, lungs and kidneys show no significant histologic changes. Tissues of the liver, spleen and lymph nodes show infiltration by foamy cells (macrophages) and the intestine shows evidence of peritonitis.

Microscopic changes are typical of an acute exudative inflammatory reaction which changes into a chronic erosive synovitis. Initial changes are observed as early as five hours after injections. Recurrent acute phases last for 10–15 days, with vascular congestion, edema, extensive fibrin deposition and infiltration by neutrophils and mononuclear phagocytes. Not only the synovial membrane, but also joint capsule, periarticular tissue, tendon sheath, muscle bundles, and muscle attachments are affected. Over two weeks the acute phase is replaced by a chronic proliferative synovitis which destroys and replaces subchondral cartilage and bone, and which is characterized by infiltrates of macrophages, lymphocytes and neutrophils, but not lymphoid follicles, in the synovial villi. The severity and duration of arthritis depends on the dose and also the strain of bacteria employed. Group A and B type streptococci are able to induce arthritis without fragmentation, and often with a long latent period. Portions of the bacterial cell which are not able to persist in the host for long periods of time induce only transient arthritis. Animals which have streptococcal cell wall arthritis exhibit other features which are comparable to rheumatoid arthritis, including chronic microcytic anemia, anergy, reduced production of interleukin-2, reduced mononuclear cell proliferation from the spleen in response to mitogens, and responses to several therapeutic agents. Histologic studies of the earliest stages of streptococcal cell wall induced arthritis show that endothelial cells are the first to be damaged.⁴⁰

2. Immunology

The inflamed synovial tissue contains increased amounts of a number of enzymes and other factors which are also upregulated in rheumatoid arthritis, including metalloproteinases, cyclooxygenase D, cytokines, heparin binding growth factor and transforming growth factor- β , neuropeptides such as corticotropin releasing hormone, and the genes *c-myc* and *c-fos*. Experiments which show that athymic nude rats can develop acute but not chronic streptococcal cell wall arthritis show that T-cells are required for that phase. Passive transfer from diseased animals to naïve recipients is successful with T-cells. In cell culture, T-cells proliferate not only in response to Streptococcal cell wall, but also in response to extracts prepared from *Mycobacterium* species, suggesting a pathogenetic relationship between streptococcal cell wall arthritis and adjuvant arthritis.⁴¹

E. CARRAGEENAN INDUCED ARTHRITIS

1. General

Carrageenan is a sulfated mucopolysaccharide which is extracted from seaweeds, the marine algae *Chondrus* spp. and *Gigartina* spp. commonly referred to as Irish moss, or carrageen moss. Multiple spellings of the word Carrageenan appear in the literature. Carrageenan is obtained and used commercially as a thickener and stabilizer in many types of foodstuffs. Carrageenan is a member of the “gel-forming polysaccharides” because κ carrageenan gels upon exposure to potassium ion. Carrageenan has a primary structure of an alternating copolymer comprised of alternating

units of sulfated D-galactose and 3, 6-anhydro-D-galactose. The secondary and tertiary structure which accompany this chemical makeup may afford some resistance to digestion by lysosomal enzymes. The potency of carrageenan as an irritant in either acute or chronic inflammatory reactions or in its effect as an anticoagulant is dependent upon both the molecular weight of the carrageenan, and also upon its characteristic fraction, either λ or κ , with the former fraction being more potent.⁴²

2. Background

Carrageenan induced footpad inflammation is a widely used model for assaying the antiinflammatory effects of medications. Carrageenan-induced arthritis was described in the rabbit in the 1960s by Gardner, Santer and others,⁴²⁻⁴⁵ after comparison against other irritants for its arthritogenicity in the tibiofemoral joint, where 0.3 ml of 1% carrageenan is injected. Carrageenan particles are actively phagocytosed by macrophages and monocytes where they remain for long periods of time.

Besides the well-documented inflammatory effects associated with the footpad injection, numerous other effects have been reported. Effects on the kinin system include *in vitro* activation of plasmin. *In vivo*, in the rat, carrageenan injection causes intense hypotension which is attributed to not only kinin effects, but also possibly other vasoactive factors which are formed via the complement activation system. Carrageenan also has an effect on the coagulation system: unfractionated, κ , and λ fractions all are anticoagulants which are about 1/15 as effective as heparin. The mechanism for this effect is not completely clear, but may be via the activation of Hagemann factor. Carrageenan also inhibits hemolytic complement, both *in vitro* and *in vivo*.

The mechanisms of action of carrageenan are extensively reviewed.⁴²⁻⁴⁶ Many of the described characteristics of carrageenan are indeterminate because the molecular structure of the carrageenans varies from year to year and also from harvest to harvest, and presumably from species to species. The κ and ι fractions of carrageenan have been demonstrated to induce thrombosis and/or infarction of the tail or digits in mice, rats and guinea pigs. This thrombogenic effect would confound studies involving arthritis; therefore, the λ fraction of carrageenan, which does not have this effect, is recommended.⁴⁷

3. Induction of Arthritis

Carrageenan is convenient for use as an arthritogen because of its chemical structure, allowing the identification in tissue by the periodic acid Schiff reaction, its easy preparation, its resistance to degradation, and the presence of sulfate groups which could be labeled. The rabbit model was extensively characterized by Lowther and Gillard, and coworkers.^{43,48} Gardner determined that the optimal concentration of the solution was 1%. In guinea pig joints six hours after a single injection, an influx of polymorphonuclear leukocytes was observed which by 24 hours was being replaced by histiocytes and, at three days, by fibroblasts, subsiding by approximately one month. In the rabbit, arthritis induced in the joint was similar histologically and morphologically to surgical specimens from rheumatoid arthritis patients. A minimum of six injections spaced no more than one week apart resulted in the following changes: increased cellularity of synovial villi with invasion of margins of articular cartilage, loss of articular cartilage, and the formation of pannus. Fibrinoid deposits and osteoclastic bone resorption were occasionally observed. Lymphocytic perivascular cellular infiltrates were dissimilar from RA, where the infiltrate is principally of plasma cells.

4. Species/Strain Differences

As in other models of arthritis there are some strain differences in the reaction to carrageenan. A subset of Wistar rats, which are genetically resistant to the anaphylactic reaction produced by dextran, failed to react to carrageenan.

5. Comparison with Other Models

The carrageenan injection model of arthritis in the rabbit offers the practical advantages of simplicity and reproducibility. Repeated injections maintain the arthritis but do not exaggerate the local response, and the lungs, liver and other viscera are unaffected.^{42,46} After five weeks of biweekly carrageenan injection, the inflammatory cellular response results in a villous hypertrophy of the synovium with perivascular collections of lymphocytes and large macrophages with a follicular appearance, and marginal cartilage erosion. Similar evolution of arthritis was reported in the specific pathogen-free pig; however, villus hypertrophy, hyperplasia of the synovium and the presence of fibrin was less than observed in the rabbit.⁴⁹ The model has been utilized in the rabbit for numerous orthopaedic research projects.

III. MECHANISTIC APPROACHES TO MODELS OF ARTHRITIS

A. CYTOKINE-INDUCED ARTHRITIS AND INHIBITION WITH ANTI-CYTOKINE ANTIBODIES

Controlled studies of cytokines in animal arthritis are of particular interest because, while upregulation of several pro-inflammatory cytokines in RA is well-known, mechanistic considerations are impossible to resolve from studies of human disease.^{50,51} The development of modern techniques which allow the isolation, preparation and use of cytokines *in vivo*, and their positive identification, has stimulated many advances in the study of pathophysiological mechanisms of arthritis. Injection of IL-1 or TNF into joints induces a transient synovitis in normal animals and worsens arthritis in several models of arthritis.⁵¹ Several cytokines of interest in the pathophysiology of arthritis and in bone metabolism (eg IL-1, TNF, TGF- β) which have been studied with mutation-based approaches are reviewed in Ryffel⁵³ and Brennan.⁵²

B. MUTATION-BASED APPROACHES

It has become feasible to manipulate both cellular makeup and cytokine makeup *in vivo*, in the same animal, an approach which has numerous advantages. A number of natural and induced animal mutations afford the opportunity to study how the omission (a.k.a. “gene-knockout”) or addition of a particular gene or its transcript affects the evolution of arthritis. These “transgenic” animals (having an altered, but stable genotype arising from a defined genetic manipulation²²) have been employed in much work associated with arthritis, but little with an orthopaedic approach to arthritis.

Spontaneous “knock-outs” include several strains of osteopetrotic animals (mice, rats, and rabbits) which lack M-CSF (macrophage colony-stimulating factor), and thus demonstrate abnormal bone development. W/W and steel mouse strains have abnormal apoptosis (timed cell death), resulting in lymphoproliferative disease. Other mutations which involve extensive lymphoid abnormality are the aly, nude and SCID (severe combined immunodeficient) mice.⁵³ Specific gene deletions to develop “knock-out” strains (also symbolized in the literature by “-/-”) can be contrived by disrupting genes (homologous recombination) in embryonic stem cells and injecting the cells into blastocytes.⁵³

Among natural mutations, one widely used model is the SCID mouse. Recent work implanting human cancellous bone under the skin of SCID mice, demonstrates that osteoblasts remain viable, forming new bone which persists.⁵⁴ Also, human synovial membrane from RA patients, implanted into the joint of SCID mice induces arthritis.⁵⁵

IV. EVALUATION METHODS

Evaluation of the specimens from animal models are covered in detail in Chapters 6 through 12. The general principles of evaluation apply as well to animal models of rheumatoid arthritis, but

certain methods developed specifically for evaluation of the animal models are discussed briefly below. The reader is directed to the appropriate references for detailed information.

The health of an animal in which inflammatory arthritis is being induced can be monitored by simple methods such as the weight, activity level and quantity of feeding. Chronically ill animals will generally differ in their weight pattern from normal controls, and this may be considered in the interpretation of the results of surgical experiments.

A. BIOCHEMICAL ASSESSMENT OF CARTILAGE AND BONE TURNOVER

There are numerous biochemical tests in serum and urine which reflect the kinetics of formation and resorption of bone and the formation and degradation of articular cartilage components. As surrogates for direct measurement of bone and cartilage, biochemical tests have the advantage of permitting serial studies during the course of disease. Molecules liberated in the process of bone formation or resorption can be followed by ELISA assay in serum or urine. Most useful to describe increased bone formation are osteocalcin, procollagen peptides, and bone-specific alkaline phosphatase. For resorption, elevated levels of calcium, hydroxyproline, tartrate-resistant acid phosphatase, pyridinoline and deoxypyridinoline (products of the degradation of Type I collagen from bone) in plasma or urine are found in RA and animal models of arthritis. It must be recognized that serological measurements in general have inherent inaccuracies related to sampling error, renal function, and tissue distribution. In the case of bone, presence of some markers (for example osteocalcin) in the serum may reflect both formation and resorption, as the marker is released during both processes.⁵⁶

B. BIOCHEMICAL ASSESSMENT OF INFLAMMATORY DISEASE

The activity of inflammatory disease can be followed in animal models with the use of the erythrocyte sedimentation rate and with acute phase reactants. These tests are discussed in Chapter 8. Biochemical assessment of cytokines within synovial fluid effusions can be performed; cytokines within the joint are highly variable and reflect a complex and redundant process of inflammation. Cytokines are released from cells within joint exudates and in synovial membrane, but also from cells in marrow spaces in subchondral bone and from chondrocytes in cartilage. The cytokines implicated in bone and cartilage degradation include PGE₂, IL-1 α , 6, TNF- α and are known to be elaborated by monocytes, histiocytes, osteoblasts, T-cells and myeloid precursors. Knowledge in this field is expanding rapidly and a current understanding of the subject can be found in recent reviews.⁵⁷

C. CLINICAL SEVERITY

Scoring systems to date for clinical severity of arthritis are various and subjective. These authors know of none to date that have been formally validated. Jee et al. have recently developed a new, systematic method of assessing joint damage in adjuvant arthritis, using automated histomorphometry.⁵⁸

D. ROENTGENOGRAPHIC METHODS

Roentgenographic evaluation is useful for following the progression of arthritis non-invasively and sequentially.⁵⁹⁻⁶¹ Small animals such as the rat are anesthetized, placed in a supine position, and their hind limbs fixed with tape, one in the lateral, one in the AP position.⁶⁰ In each radiograph, the joints of the hind limbs are evaluated together for mineralization, erosions, periostitis, cartilage space, soft tissues and limb alignment.

Radiographic grading systems for evaluating the progression of arthritis require lateral radiographs of the affected limb studied at three-fold magnification. In collagen induced arthritis, radiographs are most likely to demonstrate joint space narrowing, joint space widening, soft tissue swelling, and osteopenia, and unlikely to demonstrate abnormalities in the sacroiliac joints, hips,

shoulders, elbows or axial skeleton. Osteopenia is often obscured by periostitis at the bone margins. Changes in the joint space are not reliable because of variability due to early narrowing due to articular cartilage loss, but later apparent widening related to bone changes in the central part of the articular surface.

One of the few reports validating an assessment method for describing the bony effects of inflammatory arthritis employed scintigraphy of the arthritic right compared to left knee, and found that uptake of ^{99}Tc correlated with histological findings, in antigen induced arthritis in the mouse.⁶²

Bony changes observed in collagen induced arthritis differ from those of antigen induced arthritis, and periostitis, which is observed in several animal models of inflammatory arthritis, is rarely observed in rheumatoid arthritis, with the exception of juvenile onset disease.

E. NECROPSY: GROSS EXAMINATION

After euthanasia specific gross anatomical findings should be recorded. The presence and grade of joint effusions, fibrinoid debris and “rice bodies” in the synovial fluid, presence and grade of synovitis, area and depth of articular cartilage and bone erosion can be recorded as indices of joint damage. Capsuloligamentous distention and joint instability are also relevant findings in inflammatory arthritis.

F. HISTOMORPHOMETRY: ASSESSMENT OF BONE TURNOVER BY *IN VIVO* LABELLING

The nomenclature of bone histomorphometry has been standardized by an international committee of specialists.⁶³ An introduction to histomorphometry theory and methods, and an extensive review of earlier work have been published.⁶⁴ Inflammatory arthritis is characterized in animal models and in human rheumatoid arthritis by abnormalities of bone remodeling kinetics (Chapter 15). These abnormalities can be identified in histological sections of undecalcified bone by *in vivo* labelling of newly formed bone with bone-seeking markers.⁶⁵ These labels are incorporated into newly deposited osteoid as it mineralizes so that formation of new mineral can be documented by the primary bone parameters, labeled surface (LS) and mineral apposition rate (MAR), and secondary parameters such as bone formation rate (BFR) can be derived. Pulsed labelling by interval injection of tetracycline, calcein green, xylenol orange and/or other dyes is a standard method of bone histomorphometry, useful in osteonal analysis of cortical bone. Uncommonly used is continuous labelling of bone with markers such as calcein delivered continuously in drinking water. Careful choice of a labelling molecule, with an appropriate administration schedule, will add greatly to the kinetic information that can be obtained through bone histomorphometry. Histomorphometry requires specialized equipment, experience and skill as it is performed on undecalcified, ground sections of bone, and because labels are sensitive to light and processing methods and can be lost from specimens. For these reasons kinetic and morphometric methods, although indispensable in certain experiments are expensive and time-consuming and are often replaced where possible by surrogate measurements.

Assessment of mineralization requires undecalcified histological sections of bone, prepared with silver stains to identify unmineralized osteoid seams on the surfaces of trabecular bone. These stains do not accurately identify gradations in mineralization, which is a gradual process requiring approximately 40 days to complete. Detailed information regarding the state of mineralization of bone can be obtained via density fractionation and by specialized electron microscopic methods.

V. APPLICATIONS OF ANIMAL MODELS

A. EVALUATION OF THE PATHOPHYSIOLOGY OF RHEUMATOID ARTHRITIS

The surgical management of rheumatoid arthritis and the other rheumatic diseases requires special attention to the specific pathology and pathophysiology of these conditions. Rheumatoid involvement

of the hip, forefoot and wrist, for example, differ in important ways from degenerative and traumatic disorders at these sites, and informed surgical management reflects an understanding of the unique conditions of joint pathoanatomy and pathomechanics and changes in the soft tissue constraints of the joints. In order to evaluate new therapeutic methods in the surgery of the rheumatic diseases, and to reevaluate old ones, it is preferable to employ appropriate animal models which share features of human disease. Although there are numerous examples of appropriate investigations in animal models of rheumatoid arthritis, some of which are described below, there are many situations where the surgeon seeking guidance has no alternative to generalizing from the results of experiments performed in normal animals or in animal models of degenerative disease. The extensive literature on models of rheumatoid arthritis in Rheumatology and Immunology journals and textbooks contains numerous useful reviews of the immunology and pharmacological management of the condition.

Animal models have been applied extensively to studies of the genetics, biochemistry and pharmacology of rheumatoid arthritis and the rheumatic diseases, but less frequently to studies of bony effects, or to the surgical management of these conditions, even if a broad interpretation of the scope of surgical management is assumed.

Some of the principal areas of orthopaedic investigation where the application of animal models of rheumatoid arthritis is useful are described below.

B. OBSERVATIONS OF BONE MORPHOLOGY AND METABOLISM — EFFECTS IN ANIMAL MODELS OF ARTHRITIS

Abnormal periosteal new bone formation is observed in several of these models adjacent to sites of severe inflammation. In antigen induced arthritis in the mouse, bone apposition at medial and lateral sides of long bones occurs in most animals.⁶⁶ Osteophyte formation appears inhibited by NSAIDs (piroxicam) and by corticosteroids (prednisolone), independent of secondary cartilage formation.⁶⁷ Osteocyte death and bone marrow degeneration (osteonecrosis) are observed in tibial subchondral bone.⁶⁷

Osteopenia is a hallmark of animal models of inflammatory arthritis and RA. Many properties of cancellous bone of the epiphysis proximal to the arthritic joint are abnormal in carrageenan induced arthritis. The turnover of bone by osteoclasts and osteoblasts is rapid, so that large increases in bone formation are insufficient to compensate for bone loss, documented as a 20% decrease in trabecular bone volume (BV).⁶⁸ The trabecular surface is largely occupied by active osteoblasts, osteoid, or osteoclasts instead of a quiescent, resting trabecular surface.⁶⁹ The net effect is a rapid remodeling osteopenia. Immobilization of a normal limb in a plaster cast as a model of disuse failed to reproduce this remodeling abnormality, suggesting that arthritis effects, not immobilization are the cause of the remodeling and osteopenic abnormality.⁷⁰ Fractal analysis of cross-sectional images shows that the normal anisotropy or organizational arrangement of the subchondral trabecular bone is modified in arthritic specimens.⁷¹ Recent histological work suggested that two bisphosphonates are osteoprotective in carrageenan induced arthritis^{72,73} and also may protect the articular cartilage surface⁶⁹ from inflammatory arthritis-induced destruction. The stabilizing effects of bisphosphonates on subchondral trabecular bone and on metaphyseal and diaphyseal cortex in inflammatory arthritis (discussed in Chapter 15), are of potential interest to the arthritis surgeon. Bisphosphonates may also affect bone marrow fibroblasts and lymphocytes which are altered in carrageenan induced arthritis. Alterations in bone marrow have also been observed in adjuvant arthritis,⁷⁴ collagen induced arthritis,⁷⁴ and antigen induced arthritis,⁶⁶ as well as in RA.^{75,76}

C. FIXATION OF PROSTHETIC IMPLANTS

The fixation of orthopaedic joint implants to bone in patients who have inflammatory arthritis raises specific issues of the bone-prosthesis bond in a condition characterized by abnormalities in bone strength, microanatomy and remodeling kinetics.

Since the late 1970s fixation of metal joint prostheses to the skeleton without the use of acrylic cement has become an option in the knee and the hip, and more recently in the shoulder, elbow, hand and ankle. The fixation of prostheses by this method depends for initial stability upon an interference fit, which requires impaction of an implant with favorable contours into a bone surface precisely prepared within a joint. Beyond the brief phase of initial press-fit stability, which may require protection from weight-bearing, fixation of the implant requires ingrowth of host bone into asperities on the surface of the prosthesis, fabricated by special processes. Ingrowth prostheses have been prepared by bonding fiber metal pads, by sintering metal beads, by arc-bonding of titanium and by other methods.

Extensive research has been devoted to identifying the suitable types of metal surface, especially the size and configuration of voids, that promote rapid and stable bone ingrowth for long-term prosthetic fixation. Another relevant factor is the metallurgy of the implant: titanium and titanium alloys, for example, have been demonstrated in general to promote bone ingrowth compared to cobalt/chromium alloys and stainless steel.

However, there has been less effort to determine the effect of the other side of the prosthesis-metal bond, the host bone which serves as the bed for implantation. This is particularly relevant in rheumatoid arthritis, one of the common indications for joint replacement in the knee, shoulder, and elbow and also the hip. Bone anatomy, strength and remodeling kinetics are key to initial and long term fixation of implants, and the effects of the specific abnormalities of bone in rheumatoid arthritis require elucidation.

The authors have extensively described the abnormalities in juxtaarticular cancellous bone anatomy, remodeling kinetics, and mineralization in the carrageenan injection model of inflammatory arthritis in the rabbit, characterized by osteopenia, rapid remodeling and incomplete mineralization.^{68,77} These osteopenic features are discussed in greater detail in Chapter 15. Using a similar model in dogs, characterized by a 20% reduced bone density measured by CT densitometry, Söballe et al. found, after four weeks, a diminished bone ingrowth into titanium alloy porous-coated cylinders implanted into the distal femoral condyle, whereas ingrowth into hydroxyapatite-coated porous cylinders was not significantly different from control, non-arthritic animals. Assessment of shear strength demonstrated a corresponding decrease in the titanium, but not the hydroxyapatite-coated implants. The authors conclude that ingrowth into porous implants was impaired in osteopenic bone in inflammatory arthritis, for titanium alloy implants, but not for hydroxyapatite-coated implants.^{78,79} Similar findings were reported by Sennerby and Thomsen⁸⁰ who studied bone ingrowth onto threaded pure titanium implants in an antigen induced arthritis model. Ingrowth of bone onto pure titanium implants in arthritic rabbit tibiofemoral joints was diminished compared to control animals.⁸⁰ However, Branemark and Thomsen reported in 1997 that bone apposition and biomechanical evaluation was not impaired in collagen-induced arthritis.⁸¹

Friedman et al. compared bone apposition and ingrowth to implant surfaces and shear strength in a pushout test for cylinders of a pure titanium beaded surface, in the same surface coated with a 50 mm hydroxyapatite (HA) coating and in a grit-blasted, non-beaded titanium alloy surface with HA coating, in rabbits with carrageenan-induced arthritis.⁸² Their findings demonstrated a significant diminution of the shear strength of the bone/implant interface in all three groups in inflammatory arthritis compared to normal, possibly due to the diminished trabecular thickness and number induced by the arthritis, observed in all groups, and a thinner layer of apposed bone, observed in the third group. However, bone apposition and ingrowth were not impaired by inflammatory arthritis in these experiments.⁸³

There is a need for further studies to determine whether medications commonly used in rheumatoid arthritis (NSAIDs, immunosuppressive and antimetabolic agents) would interfere with bone ingrowth or apposition to orthopaedic implants. Whereas there are reports on the effects of indomethacin and methotrexate into porous metallic implants in normal animals, similar studies in models of inflammatory arthritis would be useful to determine how, in the presence of altered bone

remodeling in inflammatory arthritis, drugs known to affect bone formation and resorption affect cementless prosthetic fixation.

D. SYNOVECTOMY

The role of synovectomy in the management of inflammatory arthritis has been controversial for 40 years. Surgical synovectomy of joints was introduced to the management of RA by Vainio and Laine, in the 1950s in Heinola, Finland. Other methods of synovectomy have been applied, including chemical ablation of the synovium with osmic acid, radiosynoviorthesis or radiosynovectomy by injection of radioisotopes of gold, yttrium, rhenium, and strontium, and by less invasive means of surgical synovectomy utilizing the arthroscope. The original focus of joint synovectomy was the knee, but most of the other joints have been treated by synovectomy.

There would be little disagreement among clinicians who treat rheumatoid arthritis that thickened synovial tissue should be removed by the surgeon during surgical procedures performed for other, independent indications. During any surgical procedure for rheumatoid arthritis, such as wrist fusion, total knee arthroplasty, forefoot reconstruction or reconstruction of swan neck deformity, the surgeon will remove synovium with the goal of improving the mechanical function of the joint, diminishing effusion and possibly relieving a source of chronic pain.

The value of synovectomy as an independent operative procedure, performed for indications based on the benefits of synovectomy alone, has been more controversial. A clinical review of synovectomy is beyond the scope of this chapter, but there is evidence that synovectomy has value in relieving pain and improving function in certain sites, but that no effect in retarding the anatomic and radiological progression of the disease has been established.

Animal models have been applied to the controlled study of the effects of synovectomy. Chinol et al.⁸⁴ utilized an antigen induced arthritis model to evaluate the suitability of ^{153}Sm - or ^{186}Re -labeled hydroxyapatite as a radiation synovectomy agent. Low leakage rates and satisfactory distribution through the joint were demonstrated. ^{186}Re sulphur colloid also appeared to have satisfactory joint retention in a similar antigen induced arthritis model.⁸⁵ In a controlled trial, holmium-laser arthroscopic synovectomy was compared to surgical synovectomy by arthrotomy with a further sham control, in a model of antigen induced arthritis.⁸⁶ The laser synovectomy appeared to result in less capsular fibrosis than the open surgical method. Reichel and Weber reported that synovectomy resulted in a reduced exudation of plasma proteins into the knee joint, compared to preoperative controls, in an arthritis model induced by intraarticular injection of human IgG complex in immunized rabbits.⁸⁷

The carrageenan injection model of synovitis in the horse was utilized to evaluate pretreatment with ketoprofen and phenylbutazone in acute joint inflammation. Phenylbutazone was more effective than ketoprofen in reducing lameness, joint temperature, synovial fluid volume and synovial fluid PGE_2 .⁸⁸ Kim et al. reported that continuous passive motion of the knee in antigen induced arthritis, was associated with significantly greater joint swelling, synovial effusion and histologic synovitis scores compared to immobilized arthritic knees. After six weeks, however, articular cartilage was better preserved in CPM-treated than immobilized knees, as measured by articular cartilage erosion and loss of cellularity.³⁷

E. SOFT TISSUE JOINT CONSTRAINTS IN RHEUMATOID ARTHRITIS

A typical characteristic of rheumatoid arthritis is laxity of ligaments supporting joints, and capsuloligamentous distention resulting from synovial effusion, damage to the cells, collagen and proteoglycans of ligaments and suppression of the biosynthesis of type I collagen. This problem contributes greatly to the morbidity of rheumatoid arthritis by creating clinical joint instability, and also by contributing to the destruction of the articular cartilage surface, which depends upon normal articulation of the joint components.

Investigations of the biochemistry, structure and biomechanics of ligament in joint injury models and in models of degenerative or posttraumatic arthrosis have been published. We have not been able to locate published reports of studies of the deficient soft tissue restraints of joints in models of inflammatory arthritis.

Considerable morbidity in RA is related to the involvement of sheaths, with effects on the tendons. A model of tenosynovitis is described by Cooke.⁵ In this model, 0.5 ml. of 2% BSA in PBS is injected into the tibialis anterior tendon, an intrasynovial structure which is easily accessible. Within 48 hours, an acute Arthus reaction occurs, with characteristic hemorrhage, hypertrophy of the sheath lining, and influx of polymorphonuclear cells, followed by phagocytic cells and necrosis. The reaction as shown by localization of antigen within the tendon, subsides over two weeks, or six weeks, if challenge is used.

F. BONE STRUCTURE AND STRENGTH

Rheumatoid arthritis patients are at a markedly increased risk of fracture⁸⁹ which is devastating to the RA patient when it occurs in the proximal or distal femur.⁹⁰ Biomechanical failure at articular surfaces of bones (collapse of joint surfaces, weak prosthetic fixation, difficult juxtaarticular metaphyseal fractures) and in the diaphyses (fracture of long bones during or after prosthetic arthroplasty) contribute to the morbidity of RA. Extensive studies on the morphology, remodeling kinetics, mineralization, microstructure and mechanics of bone have been performed in animal models of rheumatoid arthritis, and are discussed in Chapter 15. These studies are relevant to a number of orthopaedic issues including prosthesis selection and fixation, fixation of fracture implants including screws, understanding fracture risk in RA patients and developing strategies to reduce that risk.

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Part V

Animal Models of Joint Replacement and Related Conditions



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20 Animal Models for Studying Soft Tissue Biocompatibility of Biomaterials

John A. Jansen

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I. INTRODUCTION

Prior to their clinical use biomaterials for surgical implants have to be tested on their biocompatibility. The purpose of this biocompatibility assessment is to exclude a potential toxic and carcinogenic effect of the material. Such a biocompatibility evaluation can occur at three levels,¹ i.e. (1) initial tests; cytotoxicity and mutagenicity; (2) secondary tests; and (3) usage tests.

The initial tests are mainly *in vitro* evaluation procedures. For the secondary or usage tests experimental animals are used.² Secondary tests are employed as a screening method for the local *in vivo* compatibility of materials for short and prolonged periods. The objective of the implant usage test is to investigate the functionality of a specific implant design. In general, for the initial secondary tests inexpensive, readily available but still relevant animal models are used. The final functionality evaluation occurs mostly in a different animal.

Considering the above mentioned, we have to notice that for orthopaedic implant materials the biological analysis not only comprises tests for bone compatibility. Also screening of the soft tissue

response is desirable, since a lot of orthopaedic materials come in contact with subcutaneous tissue, muscles, fasciae and tendons.

II. GENERAL *IN VIVO* TESTS

The interaction between an implant material and the surrounding tissues can be considered vital for the final clinical performance of implanted artificial medical devices. For example, the promotion of tissue attachment and the concomitant reduction of the highly undesirable chronic inflammatory response and fibrosis around implant materials are of main importance for the biocompatibility of biomaterials.³

Roughly, a test material inserted into tissue evokes two types of reactions.⁴ First, there is the inflammatory response to the surgical trauma. The following reaction is the tissue response on the biomaterial. Regarding the dynamic character of living tissues, animal models are an essential tool in the evaluation of the biological behavior of implant materials.

In designing an experimental protocol to study the soft tissue biocompatibility of implants, it has to be realized that the healing responses of different animals and tissues can vary considerably. The selection of an appropriate model and implantation site should be such that the obtained results are indicative for use in human subjects.⁴⁻⁶

A. SOFT TISSUE BIOCOMPATIBILITY ASSAYS

Soft tissue compatibility experiments can be performed on two levels:⁷ tests for local effects after implantation⁸⁻¹¹ and tests for systemic effects.¹² Considering the scope of this chapter, the significance of systemic reactions will not be discussed. For further information, the reader is referred to Merritt.¹³

Tests on local effects can be classified again according to their duration into short and long term. In short term or acute to subchronic biocompatibility experiments, the period of implantation is less than 30 days. In long term or chronic assays, the implantation time can continue from 30 days until about one year. The exact implantation time is always depending on the purpose of the study.

Since the early introduction of soft tissue implantation tests,¹⁴ new methods and approaches have been introduced continuously to qualify and quantify the tissue response. Characteristic for all methods is that the primary goal is to describe the severity of the inflammatory response. As reflected in the standards of the American Society for Testing and Materials (ASTM),⁸⁻¹¹ the still commonly used tool is histological and histomorphometrical evaluation of retrieved implants and tissue specimens. Suggested scoring indicators are: the general appearance of the tissue reaction and the presence of inflammatory cells.

B. COMMONLY USED ANIMALS

The selection of a suitable animal model for biocompatibility testing is a complex issue. It is determined by factors, like cost of animals, housing space, technical assistance and experimental objective. For soft tissue purposes, mostly rats and rabbits are used. Animals, such as guinea pigs, goats, dogs, sheep, pigs, calves and monkeys are also used.¹⁵⁻¹⁹

The advantage of rats is their availability and low cost. Further, breeding programs have resulted in rat species with almost similar intrinsic biologic properties. A disadvantage of the rat model is that the metabolic and wound healing properties are significantly different from bigger animals. We have to emphasize that this can endanger the correct extrapolation of the obtained results. Another problem with rats and all other rodents is that they can only be used for implantation studies shorter than six months. After about 6–8 months of implantation there is the risk of accidental

induction of tumors.²⁰ Consequently, for long term studies other animal models like rabbits have to be used. An additional advantage of the rabbit is that more implant specimens per animal can be tested. This facilitates the statistical design of the experiment and reduces the effect of interanimal variance. Further, bigger animals also allow the use of larger implants.

Although a wide variety of different animal species can be used for soft-tissue biocompatibility tests, it is better to stick to one or two animal models, only a few implantation sites and preferably one operator. The advantage of such a procedure is that an enormous amount of experience is obtained, which assures a good reproducibility and a high intralaboratory validity. This enables a comparative evaluation between different experiments. A disadvantage is that the results of the experiments are hard to extrapolate (to other operators, animals, laboratories, etc.).

III. FACTORS AFFECTING WOUND HEALING

Despite the use of standardized protocols and appropriate animal models, the investigator has to be aware of the fact that there are a lot of other variables which can affect the final tissue response. These influences can be of biological or experimental origin, such as surgical fluctuations (presence of microorganisms, size of incision), health or general condition of the animals (the occurrence of infections after implantation), social behavior of the animals (biting, grating), local properties of the implant site (among species, presence of subcutaneous fat), and implant characteristics (shape, porosity, mechanical properties).

A. WOUND AND REPAIR PROCESS

If the integrity of soft tissue is disturbed, e.g., by trauma or surgery, the physiological mechanisms of wound healing start. Two phases in wound healing can be distinguished, i.e. the inflammatory phase and the repair phase.²¹ Sometimes even a third intermediate phase, called the proliferative phase is described.²² In summary, during the inflammatory phase which takes about three days, the following events occur: at disruption of the integrity of the tissue, blood vessels are torn. Subsequently, the wound bed fills with blood from the torn vessels followed by activation of blood coagulation while platelets bind to the exposed collagen. The release of chemotactic substances by platelets, the activation of the complement system by exposed collagen and extracellular ATP attracts inflammatory cells. These cells, mainly polymorphonuclear granulocytes and monocytes differentiate into macrophages, which start to ingest fragments of injured tissue. Furthermore, the macrophages release substances to stimulate replication of fibroblasts and myofibroblasts at the wound edges.

At approximately the third day, formation of collagen fibres by the fibroblasts becomes histologically visible.²² In addition, a network of capillaries is formed to provide oxygen to support the fibroblast synthesis of collagen. In front of the newly formed collagen matrix, the macrophages still continue to phagocytize the dead material hereby creating an environment for other fibroblasts to settle. This process continues until the wound is completely closed. The tissue formed is called granulation tissue. Already at the sixth day of wound healing, maturation of the collagen fibres starts. By means of collagen synthesis and lysis, remodelling of the collagen network occurs. Meanwhile, myofibroblasts are responsible for wound contraction, hereby reducing the wound surface. Finally, the number of cells will decrease, leaving scar tissue behind. The functional characteristics of this newly formed tissue are less effective compared to the original tissue. The wound strength will never reach its original value and scar tissue is nonelastic.²²⁻²⁴

The presence of an implant can provide a continuous inflammatory stimulus. As a result, the acute or inflammatory phase can be prolonged.²⁵ This will be associated with an additional increase in cellular activity. If this occurs, then also the repair phase will be noticeably delayed and enhanced. The change in timescale and extensivity of wound healing and repair processes are determined by the biocompatibility of the used implant material (Figure 1).

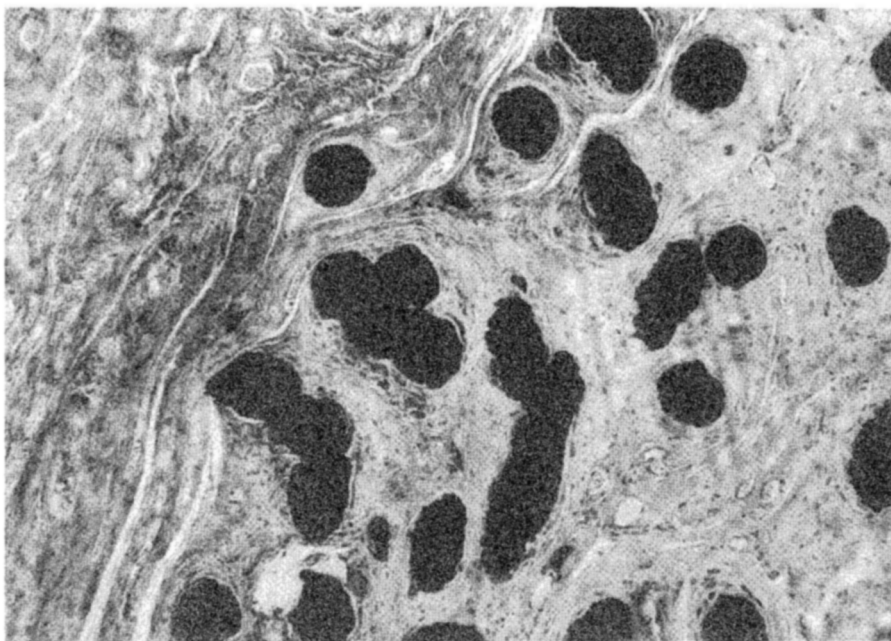


FIGURE 1. Histological section showing the soft tissue response to a porous titanium fibre mesh implant at three months after subcutaneous implantation. The used material evokes a very moderate tissue response, characterized by the presence of a thin fibrous capsule surrounding the mesh, and connective tissue ingrowth into the porosity of the mesh.

B. ANIMAL CONSIDERATIONS

Although the basic process is similar, wound healing can still vary between animal species. Gangjee¹⁸ showed that there are quantitative differences with regard to subcutaneous connective tissue response between rabbits, goats and dogs. In his study at all experimental periods (10, 15, 20 and 30 days) the number of giant cells and polymorphonuclear leucocytes around implants was highest in the rabbit and lowest in the goat. The degree of fibrous capsule maturity showed an inverse relationship. It was highest in the goat and lowest in the rabbit. Therefore, conclusions about biocompatibility tests have also to be related to the experimental animal used. Depending on the final application of the investigated biomaterial, it can even be suggested to repeat the experiment with another type of animal.

Further, the location or tissue, in which the implant is placed, can contribute to the wound healing. Picha²⁶ observed that when rough surfaced implants are placed close to fatty tissue, the tissue response will be completely different from similar implants placed in a completely muscular or fibrous tissue bed. McGeachie²⁷ inserted titanium and stainless steel wire into mouse leg muscles. Morphometric analysis showed no difference in muscle reaction between the two metals. Since these results did not corroborate with other studies in which titanium and stainless steel implants were placed in a subcutaneous position, he suggested that probably the skeletal muscle of a mouse has a high tolerance for foreign materials.

Considering the above mentioned, the conclusion appears to be justified that materials only have to be tested in the environment in which they finally will be applied. This is confirmed by Semmelink,²⁸ who describes the induction of granuloma and plasma cell formation after the subcutaneous implantation of β -whitlockite particles. In contrast, in an osseous environment bone formation is observed without an immuno-response after only one week.

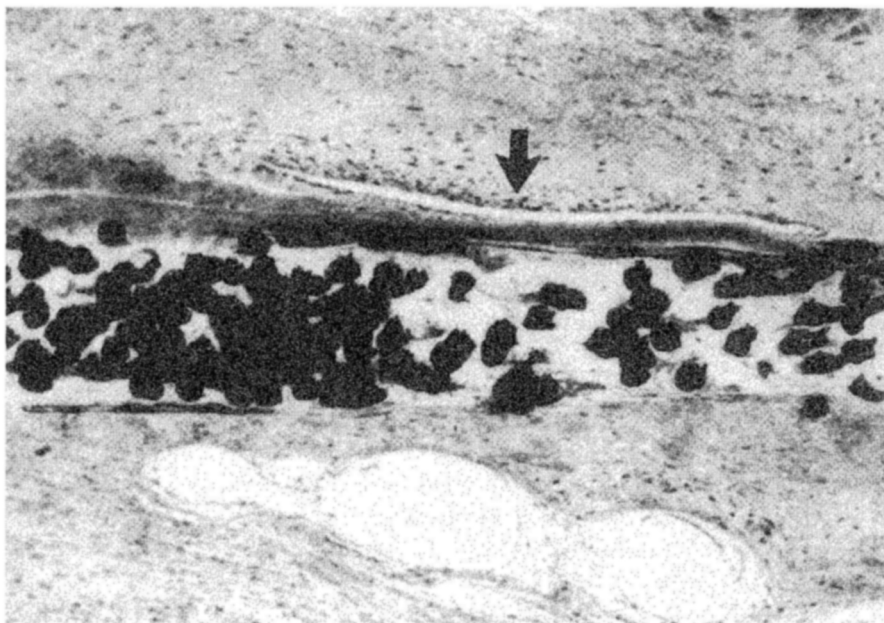


FIGURE 2. A hair (arrow) is visible in the interface between implant and surrounding fibrous tissue capsule. The hair was introduced during the surgical procedure.

C. SURGICAL CONSIDERATIONS

Surgical technique is an important contributing factor in the tissue acceptance of an implant. For example, a careful surgical technique has to be used to limit the damage to the tissue. In addition, implants which do not fit properly into the created space can cause an intolerable pressure on the surrounding tissue structures. This can lead to necrosis and subsequent failure of the implant. Further, it is known that glove powder can create histological complications including serum accumulations. Consequently, it is necessary that the surgical gloves are washed every time before the implants are touched. In addition, provisions have to be met which prevent the possible ingress of hairs or other debris into the implant pocket, like the use of special introducers for the insertion of the experimental specimens (Figure 2).

Another point of concern is migration or mobility of the implants after implantation. It has even been suggested that implants, after insertion, have to be fixed in position with sutures.²⁹ Whether this is true is difficult to say, since the suture material itself also will evoke an inflammatory reaction. On the other hand, the surgeon has to make a tissue bed in which the sample fits as well as possible. Overextension has to be prevented.

Also care has to be taken in the handling of the animals after implant placement. When implants are placed in the backs of animals, these animals should be treated carefully and not handled at the dorsum skin when they are taken out of their cages.

A final question which arises is the use of antibiotics. To prevent infection, the best approach is to use sterile surgical procedures.² Only when this is impossible, the use of antibiotics can be considered. However, it is important to note that antibiotic treatment can also interfere with the final healing response. Therefore, if given, they should be administered for as short a period as possible. A strict standardized pre-and postsurgical administration protocol, like that used for human patients, is advisable.

D. MATERIAL PROPERTIES

When designing a biocompatibility test, one must consider properties other than the physico-chemical characteristics of the implant which can influence the tissue response. For example, certain geometrical properties of the implant specimen, like shape, size, and surface topography, are known to affect the tissue reaction.³⁻⁵ Therefore, care has to be taken to standardize the geometrical features of the implants for each experiment.

The implants also have to be cleaned carefully after their preparation. Any foreign material (chemical matter, debris, etc.) left can alter the tissue response. For polymeric materials a good post-preparation cleansing procedure is first washing in 10% Liquinox solution (Alconox Inc.). Thereafter, the specimens have to be rinsed, cleaned ultrasonically for 30 minutes in a 1% Liquinox solution and given two 15 minute ultrasonic rinses in distilled, deionized water. Subsequently, they have to be given a Soxhlet rinse for 12 hours in distilled, deionized water. Finally, the substrata can be air-dried and sterilized. A sterilization process has to be used that does not change the polymer. For metallic implants, ultrasonic cleaning in 100% ethanol to remove any loose particles, is mostly sufficient. Again, the sterilization procedure has to be selected carefully, since sterilization is not always as clean as supposed.³⁰ Also the packaging of the specimens after sterilization is important. Especially, in case of rough materials, particles of the wrapping material can stick and be maintained on the specimen surface.

The mechanical properties of the implant material are also important.² Similar to bone implants, a mismatch in the mechanical properties between the implant and the surrounding tissue will result in an inadequate stress transfer and distribution at the interface. This can result in a completely different tissue response, i.e. a thicker versus thinner fibrous capsule and more versus fewer inflammatory cells at the implant-tissue interface. Consequently, specimens within one experiment must have similar mechanical properties. Otherwise, the results will be distorted.

IV. COMMONLY USED IMPLANT MODELS

The overall objective of soft-tissue biocompatibility assays is to determine the *in vivo* behavior of materials that are inserted for short or prolonged contact with tissue. Testing of the biological properties is mostly performed by inserting the materials into the subcutaneous or muscle tissues of experimental animals. For some specific applications, like the evaluation of materials used for the fabrication of leads, drains and external fixators, percutaneous implant models also can be used.

A. SUBCUTANEOUS IMPLANT MODELS

For subcutaneous testing, the dorsal subcutis of the experimental animal is the preferred location.¹¹ Before implantation, the animals are anaesthetized and placed in ventral recumbancy. Then, the back of the animal is shaved, scrubbed with Betadine®, and disinfected with iodine. Paravertebral, between the scapula and the hind limb, a longitudinal incision is made on the left and right sides of the spinal column, through the full thickness of the dorsum skin. Depending on the animal type and size one or more incisions can be made. Subsequently, lateral to the incision a subcutaneous pocket between skin and muscle fascia is created by blunt dissection with a scissors. One implant is inserted in each pocket. When more implants are placed per animal, contact between the specimens after insertion has to be prevented. Finally, the wound(s) are carefully closed with resorbable sutures. Depending on the animal species and housing of the animals, various kinds of suture techniques can be used. For example, in our laboratory we use an intracutaneous suturing technique when the animals are housed together.

At the end of the experiment, the animals are sacrificed and the skin is shaved again. Subsequently, an incision is made through the skin lateral to the implants. Then, the implants are exposed by retracting the skin from the underlying muscle tissue and the implants with their surrounding

tissues and the overlying skin are excised. Skin tissue always has to be included into the retrieved sample. This facilitates the final histological comparison between normal and regenerated tissue. For this reason, the animals are shaved after euthanasia. Without complete hair removal, the histological preparation of the samples is hampered, because the embedding material cannot easily penetrate into the sample. Directly after retrieval, the tissue specimens are fixed in 10% buffered formalin. After fixation, the samples can be trimmed to remove excess tissue.

Mostly the specimens are subjected to routine light microscopical examination. When more sophisticated evaluation techniques are used, perfusion fixation is used instead of immersion fixation.³¹

B. INTRAMUSCULAR IMPLANT MODELS

A lot of orthopaedic devices will come in contact with skeletal muscles. Although muscle tissue is highly vascularized, it shows less regenerative capability.⁶ In addition, due to intrinsic stress factors related to motion of the muscles, the biocompatibility response of implant materials placed in muscles can differ from subcutaneous tissue. Paravertebral and gluteal muscles are the test sites of first choice for intramuscular implant models.⁸

After anesthesia, the skin over the dorso-lumbar or pelvic regions is shaved and disinfected with Betadine and 75% ethanol. A longitudinal incision is made through the skin and the paravertebral gluteal muscles are exposed. The skin is separated from the underlying fascia with blunt dissection. Subsequently, the fascia is dissected and a small incision is made into the belly of the muscle. An implantation site is created by further separation of the muscle fibers using blunt dissection with a hemostat or rounded scissors. After insertion of the implant, the muscle incision is closed with resorbable sutures. The last step is closure of the skin incision. Depending on the size of the animal, one or more implants can be introduced in the muscle tissue.

At the end of the experiment, the animals are killed and the implants along with a generous zone of surrounding muscle tissue are excised.

C. PERCUTANEOUS IMPLANT MODELS

Percutaneous implants can be placed at different locations. In our laboratory, we implant them into the dorsum, into the tibia and onto the cranium of various types of experimental animals, i.e. guinea pigs, rabbits and goats.³²⁻³⁴

For the testing of drains, we prefer the use of goats. For the insertion of the specimens, the animals are anesthetized and the region distal to the costal ridge is shaved, washed and disinfected with iodine. A longitudinal incision is made parallel to the spinal column. Lateral to this incision a subcutaneous pocket is created by blunt dissection with scissors between the subcutaneous fat layer and the musculus obliquus abdominis externus. Centrally in the subcutaneous pocket, the muscle is cleft parallel to the muscle fibers over a distance of about 0.5 cm and a small tunnel is created by blunt dissection. Then, the drain tube can be inserted in this tunnel. Thereafter, the wound is closed using resorbable sutures. To prevent postoperative damage of the wound site, we stable the goats separately with their heads fixed between two vertical bars to prevent the animals from manipulating the percutaneous tubes.

In experiments where percutaneous leads have to be used, percutaneous implants are inserted into the dorsum and on the cranium. The implants are flange-shaped to obtain sufficient subcutaneous stabilization and fixation.

For the cranium implants we prefer the rabbit as experimental animal. After anesthesia, shaving and disinfection, a longitudinal incision is made on the rabbit's skull, approximately 3 cm caudal of the orbita. After exposing the os frontale, the skin is bluntly undermined and a subcutaneous pocket is created. The flange-shaped implant is created and the skin is sutured. Besides separate housing, no special measures have to be taken to prevent damage to the device or percutaneous passage.

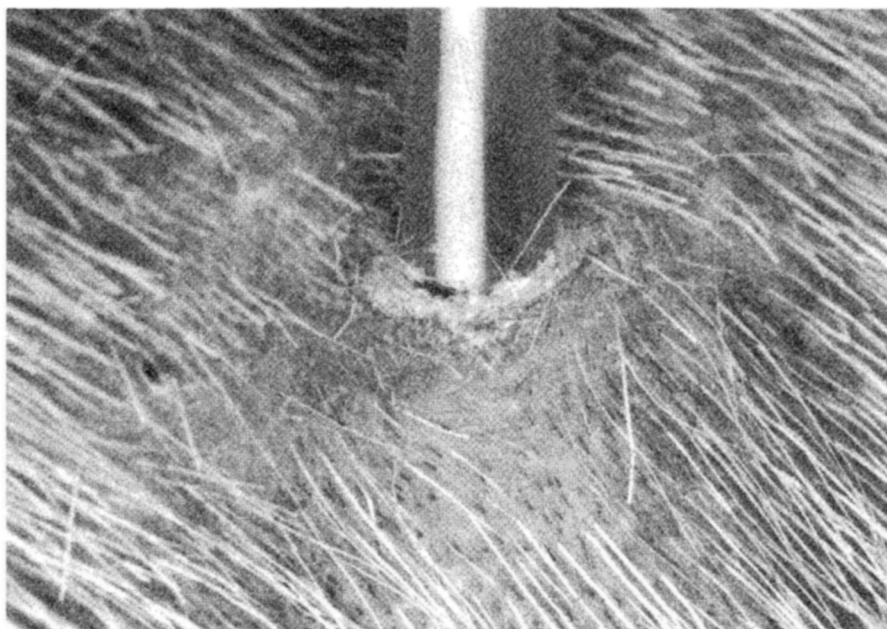


FIGURE 3. A macroscopic picture of a successful percutaneous lead, inserted into the dorsum of a goat, after four months of implantation.

For the dorsum implants we either use rabbits or goats. The surgical technique for placement of the implants always consists of two stages. During the first session of this two-stage procedure, only the subcutaneous part of the percutaneous device is inserted. For installation of the subcutaneous component, a subcutaneous pocket is created lateral to the spinal column. After closure of the wound, the subcutaneous implant is left to heal for a period of at least six weeks before the second stage surgical procedure is performed. At the second session, a small incision is made through the skin over the implant. Subsequently, the percutaneous part of the implant is fixed in the subcutaneous. For this purpose, the subcutaneous component is provided with a special holding element. The last step is closure of the incision with one straight suture. When goats are used, the same protective measures to prevent mutilation of the exit-site have to be taken as described earlier (Figure 3). Rabbits only have to be housed in separate cages.

For the testing of external fixation devices we use the tibia as implantation site. To install the devices a longitudinal incision is made on the medial surface of both legs. After exposing the bone, a hole is drilled through the medial cortex, the medulla and the lateral cortex of the tibia. The implants are inserted in the tibia, so that they clearly protrude above the skin surface, and the incisions are closed. The amount of skin protrusion is determined by the size of the experimental animal.

V. COMMON EVALUATION METHODS

At the end of the implantation period, the experimental animals are sacrificed and the implants with their surrounding tissue retrieved for further evaluation. First inspection consists of a gross examination of the specimens on abnormalities in tissue appearance. Thereafter, further processing is necessary for the histological and histomorphometrical evaluation of the implant-tissue specimens.

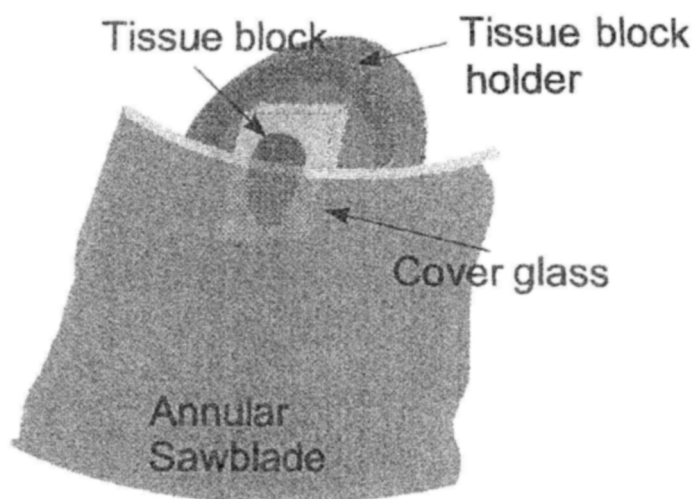


FIGURE 4. Schematic drawing of the sawing technique showing the coverglass fixed to the tissue block. A thin section is cut.

A. LIGHT MICROSCOPICAL PREPARATION

For the correct light microscopical sectioning of implant-containing specimens, only two techniques are suited: (1) the “sawing-grinding” technique as developed by Donath³⁵ and (2) the modified inner circular “sawing” technique as developed by van der Lubbe and Klein.^{36,37} Both methods require embedding of the samples in methylmethacrylate before sectioning.

The equipment necessary for the sawing-grinding method (Exakt-cutting-grinding-system®, Exakt Apparatebau, Germany) consists of a precision guided, diamond-coated band saw and an automatic grinding machine. With the band saw, a first cut is made through the polymerized block. On the exposed tissue-implant surface, a microscope slide is glued. This slide is mounted again in the band saw using a vacuum slide holder. Subsequently, a planoparallel section is cut of a thickness between 50–200 μm . This section is thinned down to a thickness of 5–10 μm with the grinding machine. Finally, the section is stained. For the staining, all the usually employed staining procedures for plastic embedded tissues can be used (see Chapter 7).

For the modified inner circular sawing technique, a horizontal innerlock saw microtome (Fijn-metaal Techniek, Amsterdam, The Netherlands) is used with many adjustments, i.e., freedom of movement of the saw blade, balanced rotation mechanism, thickness of sectioning. To prepare sections, the polymerised tissue/implant block is fixed in the specimen holder and a first cut is made to expose the implant surface. After staining the exposed specimen surface with basic fuchsin, Giemsa or methylene blue, a glass coverslip is fixed on the sample surface with cyanoacrylate-based glue. After drying, the coverslip, with the attached tissue/implant, is sawed off the block using a 1:1 mixture of glycerine and water as cooling liquid and lubricant (Figure 4). The sections obtained have a thickness between 5–10 μm . Finally, a glass slide is glued against the section.

Using the above described methods, it is possible to make thin sections of implant material and surrounding tissue without damaging the interface. It is difficult to express an opinion regarding which method is preferred; both methods have their merits. For example, the sawing-grinding technique results in sections of very high quality. On the other hand, the sawing technique is less elaborate (no grinding or polishing) and allows the preparation of more sections of implant samples with a small diameter.

After the sectioning procedure, the obtained sections can be investigated by light microscopy.

B. HISTOLOGICAL AND HISTOMORPHOMETRICAL EVALUATION

For estimation of the soft tissue response to the implants, histological and histomorphometrical evaluation can be performed. Areas of interest for the biological evaluation of soft-tissue implants include: (1) Implant: using the above described techniques, light microscopic sections with the implant *in situ* can be made. The histological appearance of an implant can give information about the stability or degradation behavior of a material. (2) Surrounding tissue: this is the soft tissue capsule surrounding the implant. This capsule is considered to show the inflammatory and healing reaction in response to the surgical trauma and the continued presence of the implant. (3) Interface: this is the type of tissue directly adjacent to the implant surface. The nature of this tissue is determined by the chemical and physical properties of the biomaterial. (4) Interstitial tissue: in case of a porous implant connective tissue will grow into the implant. This is the interstitial tissue. The degree of ingrowth will, in addition to the chemical and physical properties of the material, also depend upon the biomechanical conditions of the biocompatibility test.³⁸

Considering these areas of interest the histological evaluation consists of a thorough description of the observed tissue reaction. For the histomorphometry, the following assessment parameters can be used: (1) Epidermal downgrowth (the distance of epidermal migration alongside the implant) and sulcus width (the distance between percutaneous component of the device and the skin). This analysis holds only for percutaneous implant models. (2) A semiquantitative and semiquantitative histological grading scale in which the histological characteristics of the surrounding tissues, interface and interstitium are evaluated by assigning scoring points. Various grading scales are available. The semiquantitative classification of the capsule frequently consists of measurement of the capsule thickness by counting the number of observed fibroblasts. The semiquantitative rating of the capsule, interface and interstitium can be based on numerically rating the tissue morphology (fibrous tissue, maturity, presence of connective tissue or fat tissue) and cellularity (presence of fibroblasts, macrophages, giant cells and other inflammatory cells).³⁹ An example of a semiquantitative rating system is given in Table 1. (3) The presence and number of blood vessels, plasma cells and inflammatory cells (macrophages, giant cells, polymorphonuclear granulocytes) in the interstitial tissue and surrounding fibrous capsule.

The histomorphometric analyses have to be performed on a sufficient number of representative sections of each implant (at least two sections per implant) and done blindly.

C. OTHER EVALUATION METHODS

Light microscopy is especially fitted to obtain in a fast and more or less simple way information about the whole tissue part containing the implant. Occasionally, a more detailed or very specific assessment of the tissue reaction has to occur.

When, for example, accurate information about the tissue changes next to implants is required, immunohistochemical analysis can be used.⁴⁰ Besides information about the local immune reactions,⁴¹ immunostaining techniques also offer a possibility to study the presence and distribution of proteins involved in soft tissue remodeling.⁴²

Evaluation of biomaterials can also be done by using electron microscopical techniques. Transmission electron microscopy (TEM) provides ultrastructural information about differentiative cellular changes in relation to a specific biomaterial. A disadvantage of TEM is that the preparation of tissue sections is very time consuming. In addition, most biomaterials are too hard to allow the preparation of ultrathin TEM sections. Often, the implant is removed. However, removal of the implant impedes a proper investigation of the tissue-implant interface. Therefore, several methods for preparing ultrathin sections containing intact implant-tissue interfaces have been explored (see Chapter 6). Also, 10 µm sections created by the inner circular "sawing" technique can be used for TEM examination.³⁷ Scanning electron microscopy (SEM) allows the spatial three-dimensional

TABLE 1
Example of Parameters Used in the Histological Analysis
of Soft Tissue Implants

Category	Number or score
General	
Section No.	Independent
Animal No.	Independent
Side	L or R
Site	1, 2, 3...
Implantation period	1, 2, 3, 4, days or weeks
Capsule Localization	
No capsule present	1
Capsule on 1 (dermis) side	2
Capsule on 1 (medial) side	3
Capsule on two sides present	4
Capsule Formation	
No capsule present	1
Loose, fibro-elastic	2
Loose, adipose	3
Loose, fibro-adipose	4
Less dense	5
Dense	6
Capsule Cellular	
— Fibroblast thickness	[1 = 0, 0<2<5, 5 <3<10, 10< 4<30, 5>30]
— Fibroblast contacting surface	1 = YES, 2 = NO
— Acute/chronic inflammatory process	1 = AC, 2 = CHR
— Severity inflammatory process	1 = none, 4 = severe
Inflammatory Cells Location	[1 = non, 2 = end, 3 = middle, 4 = 2 + 3]
— Inflammatory cells contacting surface	1 = YES 2 = NO
macrophages	1 = YES 2 = NO
giant cells	1 = YES, 2 = NO
PMNs	1 = YES, 2 = NO
plasma cells	1 = YES, 2 = NO
— Blood vessels present	1 = YES, 2 = NO
mature/new vessels	1 = MAT, 2 = NW
Capsule Surrounding Tissues	
— Acute/chronic inflammatory process	1 = HC, 2 = CHR
— Severity inflammatory process	1 = none, 4 = severe
macrophages	1 = YES, 2 = NO
giant cells	1 = YES, 2 = NO
PMNs	1 = YES, 2 = NO
plasma cells	1 = YES, 2 = NO
— Blood vessels present	1 = YES, 2 = NO
mature/new vessels	1 = MAT, 2 = NW

examination of tissue-implant specimens. Although, the occurrence of drying artefacts is a well-known phenomenon in SEM samples, the recent development of a so-called “environmental” SEM has almost completely solved this problem.

Finally, electron probe X ray microanalysis (XMRA) can be used to determine changes in elemental composition of the cells and tissues surrounding implants on a microscopic scale.⁴³ Such changes can occur due to release and accumulation of chemical trace elements from the implanted material.

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21 Animal Models of Bone Ingrowth and Joint Replacement

Dale R. Sumner, Thomas M. Turner, and Robert M. Urban

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I. INTRODUCTION

In this chapter, we review animal models related to two areas of orthopaedics: (1) bone ingrowth and (2) joint replacement. Our focus is primarily on considerations pertinent to the use of the models, with less emphasis on specific findings. The chapter, however, should be useful as an entry point to this literature. In most cases we have restricted citations to book chapters and peer-reviewed manuscripts, although in a few instances we have cited peer-reviewed abstracts. The reader should be aware that this is an active area of research and that many new results have not yet been published in peer-reviewed journals. Recent transactions from the Orthopaedic Research Society and the Society for Biomaterials should be consulted for the most recent findings.

II. BONE INGROWTH

The term *bone ingrowth* refers to the development of new bone tissue within an implant. Typically, in orthopaedics the implant receiving the new bone is a porous bone graft substitute or a porous-coated joint replacement component. Our direct experience is primarily with joint replacements and that will be the focus of our discussion, but it is thought that the same general principles that apply to joint replacements also apply to bone graft substitutes.

Research questions involving bone ingrowth include the feasibility of using various porous materials, desirable implant characteristics for bone ingrowth, the effects of interface motion and gaps, the effects of adjuvant therapies used during joint reconstruction, and means of enhancing implant fixation. Our intention is not to review these studies per se as several recent reviews are available,¹⁻⁶ but to review briefly the basics of bone ingrowth, discuss the types of models currently available and issues associated with experimental design, and then mention a few highlights from recent studies.

A. GENERAL PRINCIPLES OF BONE INGROWTH

The general principles of bone ingrowth have been identified for some time and comprehensive reviews of the tissue and cell level mechanisms are already available in the literature.^{1,4} Bone ingrowth occurs if the implant (1) is made from a biocompatible material, (2) has the appropriate porosity and integrity, (3) is mechanically stable, (4) is in close contact with the host bone and (5) the implantation site is not infected. The process is one of intramembranous bone formation and, thus, resembles gap or defect healing as opposed to endochondral aspects of fracture healing (Figure 1). From a practical point of view, the materials in commercial use are biocompatible and have the appropriate porosity, and with proper surgical technique the risk of infection is low. So, important practical issues involve mechanical stability and proximity of the implant surface to host bone.

Excessive micromotion between the implant and host bone and gaps at the interface are known to inhibit or prevent bone ingrowth. It is generally accepted that relative motion of 150 μm or more leads to failure of fixation by bone ingrowth.^{7,8} Motions as small as 40 μm appear to inhibit fixation by bone ingrowth, but interface motion of 20 μm appears to permit fixation by bone ingrowth.⁸ Gaps of 0.5–3.0 mm have been shown to inhibit bone ingrowth, with the larger gaps causing more inhibition.^{9,10}

B. BONE INGROWTH MODELS

Bone ingrowth has been most thoroughly examined in canine models, although other species have been used. Interestingly, a recent study designed to investigate the effect of ovarian function showed comparable results in canine and primate models.¹¹ Beyond this study, there is very little direct comparison of species. Based on our own experience with canine and primate models and implants retrieved from patients, it is our opinion that the process, morphology and governing factors operant in humans are well-modeled in canines.

The models can be classified in several ways, including site investigated (cortical v. cancellous bone or metaphysis v. diaphysis), loading status (nonweight bearing v. weight bearing), fit (press-fit v. gap) and host bone status (normal v. altered). The choice of model depends upon the question being asked. If one is interested in testing whether a new material will support bone ingrowth, the simplest approach is to use a nonweight bearing, press-fit model. In contrast, if one is interested in testing a new treatment thought to stimulate bone regeneration, then the most efficient model would be one in which bone ingrowth is inhibited, perhaps progressing from simple to complex models (e.g., a nonweight bearing gap model to a revision total hip replacement [THR] model).

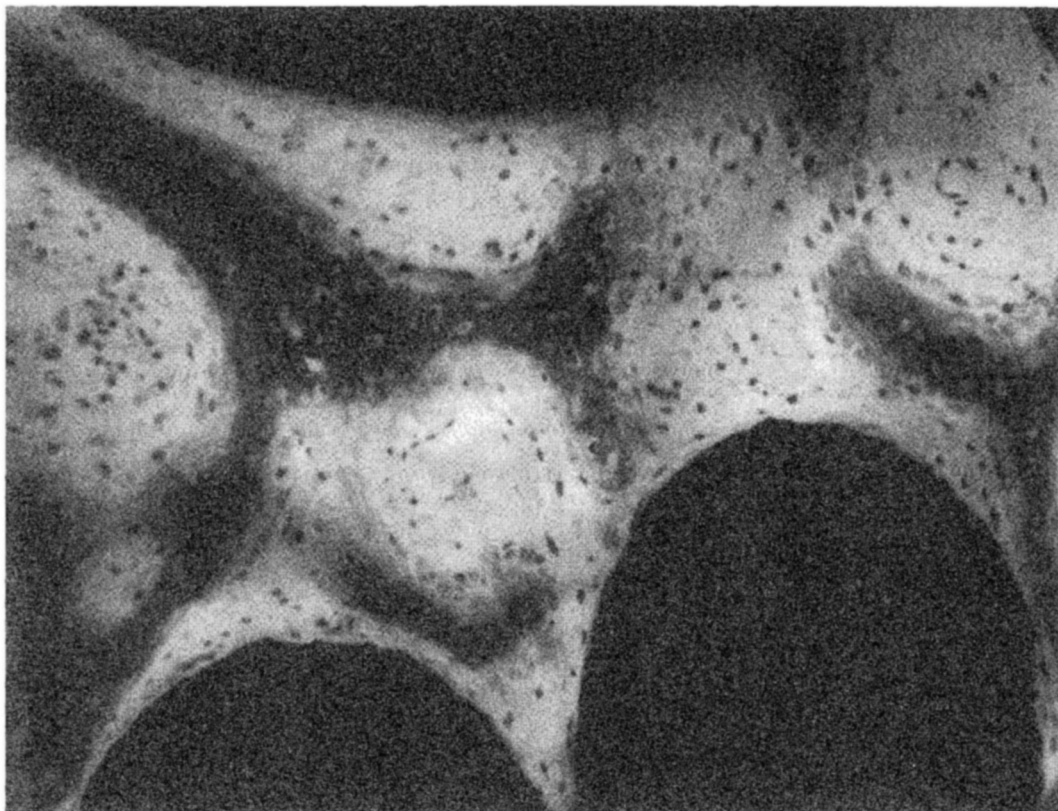


FIGURE 1. Photomicrograph of bone ingrowth at two weeks in a canine model. Note the presence of new intramembranous bone within the void spaces of the porous coating. (from Galante, J. O. and Rivero, D. P., in *Advanced Concepts in Total Hip Replacement*, Harris, W. H., Ed., Slack, Thorofare, NJ, 1985).

1. Nonweight-Bearing Models

Classically in joint replacement research, models may either be categorized as nonweight bearing or weight bearing. The nonweight bearing devices are not directly loaded, are usually implanted for short periods of study (days to weeks) and are used to study implant material or the bone-implant interface isolated from the effects of cyclic weight bearing. In general, these models are used to study implant-related issues (e.g., materials or coatings or surface modifications) or the effects of treatments that may inhibit or enhance bone ingrowth. If a material or surface structure appears promising in a nonweight bearing application, the next consideration is to test the concept under the influence of cyclic weight bearing. This generally necessitates either a segmental replacement or a joint replacement model.

Nonweight bearing models can be classified into those which are applied as a press fit or in which an interfacial gap or defect is created and they can also be classified according to placement location (Figure 2). Placement may be such that the long axis of the implant and bone are aligned (“axial” placement) or so that these two axes are orthogonal (“transcortical” placement). The axial intramedullary devices may be restricted to insertion into only the distal or proximal metaphyseal bone or through all regions of the bone, metaphyseal and diaphyseal. Thus, the shape and size of the device may afford apposition to only metaphyseal trabecular bone or to both metaphyseal trabecular bone and the endosteal cortical surface of the diaphysis.

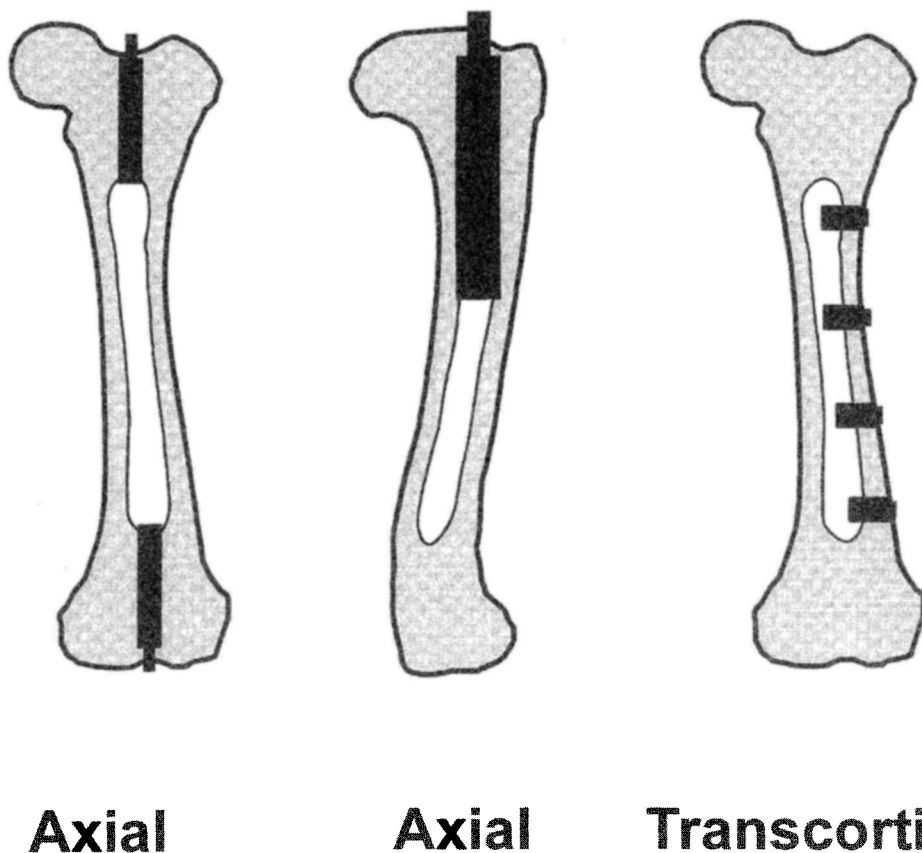


FIGURE 2. Schematic showing placement of axial and transcortical nonweight-bearing implants for investigation of bone ingrowth.

Transcortical devices are implanted at right angles to the long axis of the bone either in the metaphyseal or diaphyseal region of the bone. Thus, the transcortical implants typically are in contact with cortical bone only (in the case of most diaphyseal sites) or a mixture of cortical and medullary trabecular bone (in the case of metaphyseal sites).

The nonweight bearing devices provide the ability to isolate bone surface as a variable for study, for example, the response of endosteal cortical bone or metaphyseal trabecular bone to an implant. We believe it is most clinically relevant to include a site in trabecular bone or a site adjacent to the endocortical surface because most joint replacement implants are placed within a trabecular bone bed (e.g., the acetabular component in THR and the tibial and femoral components in total knee replacement [TKR]) or within the medullary cavity (e.g., the femoral components in THR). Thus, our bias is that axial and transcortical metaphyseal implants provide more clinically relevant information than transcortical diaphyseal implants.

Nonweight bearing devices also allow for placement of multiple implants within one animal. The test devices are frequently implanted bilaterally, with one side serving as a test material and the other side serving as a paired control. For transcortical implants, sometimes several implants are placed diaphyseally, yielding as many as six to ten implants per animal. From a statistical point of view, each animal, not each implant, constitutes a sample. Thus 10 implants in one animal yields

a sample size of one, not 10. Other aspects of sample size determination are described in the section on joint replacement (below).

2. Controlled Motion Models

A disadvantage of nonweight bearing implants is that they cannot replicate the normal load distribution from a weight bearing prosthetic device to the bone nor can they replicate the cyclic loading conditions that a weight bearing prosthetic device undergoes. Recently, this difference between nonweight bearing and weight bearing implants has been bridged to some extent by designing model systems in which the bone-implant interface micromotion can be controlled. In one model system, Søballe and colleagues developed a means to impart controlled motion at the bone-implant interface by having a plunger contact the tibia during the gait cycle.^{7,12} In another model Harris' group developed a mechanized means to control the motion of a transcortical metaphyseal implant with respect to the host bone.⁸

3. Models Communicating with the Joint

Another type of implant that bridges some of the differences between nonweight bearing and joint replacement models is the use of devices that communicate with the joint.^{13,14} For instance, this type of model has been used to investigate the migration of particles around a device interface without the compounding effects of weight bearing and component movement. Controlled motion can also be imparted to this type of model.¹⁵

4. Weight Bearing Models

Weight bearing devices have been studied most frequently in the dog; however, other species such as primates, goats or sheep have been utilized. Segmental replacement has been studied, typically centered around a diaphyseal replacement prosthetic device.^{16,17} These devices have usually succeeded in having a successful union at the proximal and distal bone-implant junctions but a lesser amount or no bony incorporation in the mid-aspect of the device.

5. Hip Replacement Models

A more common weight bearing model, particularly in recent years, has been hip replacement. This has been applied in two forms. One is a THR with both acetabular and femoral components being inserted. The other is a hip replacement hemiarthroplasty in which only the femoral head is replaced, thereby avoiding the potential complications of an acetabular component. The use of any joint replacement device in an animal model allows that device to experience the cyclic loading of ambulation both for the prosthetic device materials as well as the bone-implant interface. Although different surgical approaches have been utilized for the implantation of hip replacement components, this is a reliable model which can provide very successful clinical function provided proper implantation of the device is achieved.

Variations on the bone-implant interface have also been studied by allowing the presence of only a press fit or, alternatively, the development of defects in the bone adjacent to the implant. Thus, hip arthroplasties may be implanted as a press fit device with the components being impacted into an undersized prepared cavity or as a gap model in which control defects are developed or created adjacent to the prosthetic bone interface. These defect models have been used to test various bone grafts and bone graft substitutes.¹⁸⁻²¹

A further modification of the weight bearing prosthetic joint model is the development of revision models that replicate the bony environment developed in the site surrounding a failed prosthetic device.²²⁻²⁴ The altered bony environment includes the presence of macrophage-laden granulomas rather than bone marrow at the site of implantation.²³

6. Knee Replacement Models

Other prosthetic components, notably TKRs have also been used. TKR models are less frequently used, as only a handful of reports have been made^{25–31} compared to the large number of THR studies (see below, under the Joint Replacement section). Knee arthroplasties are more complex than hip arthroplasties because of the greater complexity of motion at the knee joint than at the hip joint.

C. EXPERIMENTAL ENDPOINTS

For studies of bone ingrowth, the experimental endpoints typically are morphological (e.g., measurements of the amount of bone ingrowth or bone formation in a gap) or mechanical (e.g., the strength of fixation of the implant to the host bone). There are various morphological instruments. Two we find useful are the “volume fraction” and “extent” of bone ingrowth. The volume fraction refers to the amount of void space within the porous coating occupied by bone and can be measured with the aid of an image analyzer and backscatter scanning electron micrographic images,³² although point counting of properly imaged and stained ground sections works just as well and in a small-scale study might be preferable. The extent of bone ingrowth is a means to quantify the topographic distribution of bone ingrowth, and can be considered a measure of consistency. Researchers have performed this observation in various ways, but the basic concept is to divide the porous coating into a number of equal size units (e.g., 1 mm fields) and then to determine how many of these fields contain bone ingrowth.^{33,34} We have also found it helpful to use measures of trabecular architecture first developed by researchers in metabolic bone disease to characterize the newly formed bone in gaps adjacent to test implants.¹⁰ Other morphological observations have been made, including the presence and thickness of fibrous tissue layers at the interface,³⁵ bone tissue kinetics,³⁶ and it can be anticipated that techniques to better understand gene expression (such as *in situ* hybridization and immunohistochemistry) will be used in the future.

Mechanical measures typically have focused on the strength of fixation.^{37,38} The interface shear strength is measured by a “pull-out” or a “push-out” test (Figure 3). Typically, pull-out tests have been used for implants placed in an intramedullary site, while push-out tests have been used for both transcortical implants and intramedullary implants. These tests are destructive since the interface is stressed to failure and the peak load is divided by the nominal surface area to calculate the strength of fixation. Recently, we have proposed a nondestructive test,³⁹ but this has not yet been performed on actual implantations. The basic concept of the test is that it should be possible to measure interface stiffness in a nondestructive way so that both mechanical and morphological observations can be made on the same specimen. Of course, it is possible to measure some aspects of bone ingrowth on an implant that has been forcibly removed from the host bone, but the interface itself is destroyed during destructive testing.

For the weight bearing models, researchers typically measure the amount of bone ingrowth as with the nonweight bearing models. However, for these models, it is more common for the mechanical stability of the implant to be measured than for the strength of fixation to be measured.^{29,31,40–45}

D. FACTORS AFFECTING BONE INGROWTH: RECENT HIGHLIGHTS

There are many factors which can affect bone ingrowth such as bone grafts and bone graft substitutes, implant surface treatments, electrical stimulation, growth factors and adjuvant treatments for conditions such as heterotopic ossification, cancer and immune-mediated diseases. Rather than review specific studies on factors affecting bone ingrowth (which have been reviewed in detail elsewhere^{1–5}), we felt it would be more useful to highlight a few recent studies.

It is generally thought that the amount of micromotion at the interface influences tissue differentiation, with stable implants permitting bone ingrowth and unacceptable levels of micromotion causing fibrous tissue formation. However, the biology of tissue differentiation at the

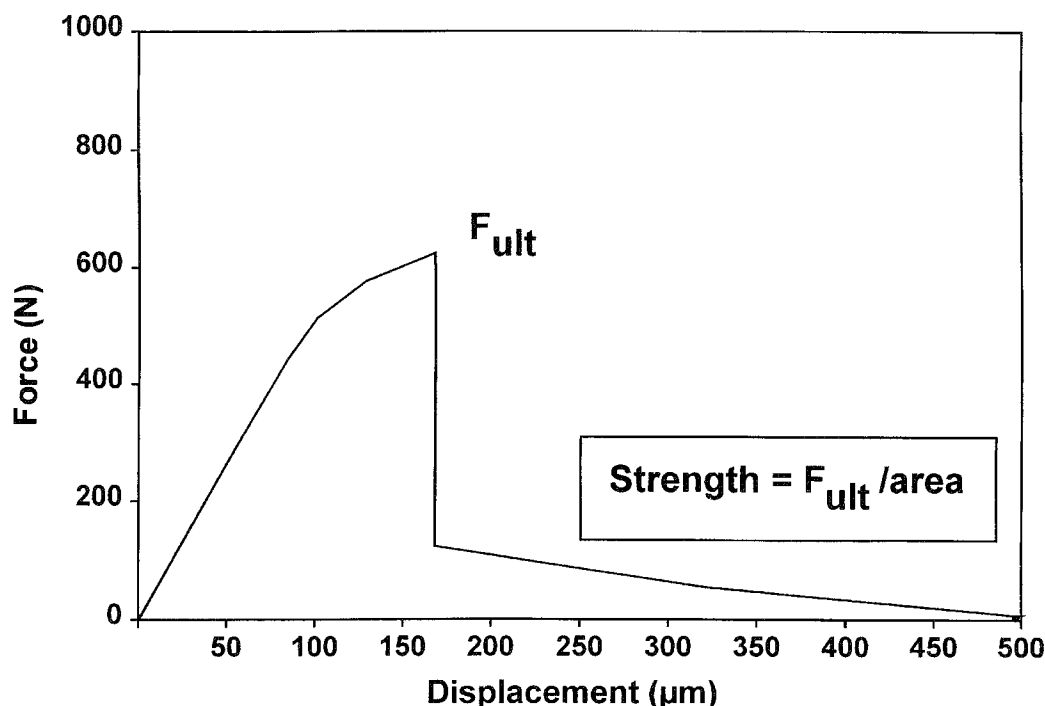


FIGURE 3. An example of a load-displacement curve from a “pull-out” test. Note that the ultimate force can be divided by the nominal surface area of the implant to calculate the strength of fixation. The slope of the curve can be used to calculate the interface stiffness if one accounts for the compliance of the experimental set-up.

interface may be more complex as evidenced by a recent canine study⁸ in which 20 μm of initial motion led to increasing interface stiffness during the course of the six week study and continuity of bone ingrowth, 40 μm of initial motion led to decreasing interface stiffness and mixed bone and “fibrocallus,” and 150 μm initial motion led to decreasing interface stiffness and a fibrous tissue interface despite the presence of some bone ingrowth. This later finding is quite intriguing because it suggests that bone differentiates, but then possibly undergoes stress fracture and replacement by fibrous tissue in the presence of excessive interface micromotion. Thus, the conventional view, that excessive motion causes direct formation of fibrous tissue, may be incorrect.

We and others have recently shown that transforming growth factor-beta (TGF- β) enhances bone ingrowth or implant fixation strength.^{10,46} More recently we have found that there is a positive effect on bone regeneration at the contralateral, non-growth factor-treated, control site as well as at the site directly treated with the growth factor.⁴⁷ We have also recently found that bone ingrowth and regeneration are enhanced with local delivery of bone morphogenic protein-2.⁴⁸ With both of these growth factors the response is dose-dependent and, in our studies, the lowest of the doses studied to date has been the most effective.

III. JOINT REPLACEMENT

As with the studies of bone ingrowth, the most common animal model of joint replacement is the canine. By far, most of the work has focused on the hip joint with only a few studies of the knee (Figure 4). The research questions are varied, but many are concerned with various aspects of cementless total joint replacement, including implant fixation and adaptive bone remodeling. In

this regard, the nature of the interface, materials and implant shape have been investigated. In addition to concern with bone ingrowth and remodeling, other areas investigated include bone cement, the type of bearing surface and wear debris. This latter topic is covered in a separate chapter in this book.

In this section, we focus on methodological issues such as species choice, experimental design, and experimental endpoints. We then briefly review studies on bone remodeling and provide a listing of various studies to provide the reader an entry into the literature. Our listing is not meant to be exhaustive, but in conjunction with some recent reviews,^{3,49–52} should be helpful.

A. METHODOLOGICAL ISSUES

The appropriate experimental design depends, of course, upon the research question(s) being asked. Many studies involving total joint replacement can be considered “feasibility” studies. In these studies the question might be, can new material X be used successfully? The first step is to perform a few trial implantations and look for obvious problems. Should none be found, the research may progress to the next stage: how does new material X compare with some standard? At this point, a number of important issues develop, including the type of experimental endpoints, the power of the experiment, and the potential influence of confounding variables.

We can’t hope to review all possible research questions so we will review here our own philosophy of experimental design, using some specific examples from our research. Recently, we performed a study to compare bone ingrowth and functional adaptation of the host bone to femoral stems varying in stiffness.⁵³ The clinical problem being addressed was that of excessive proximal femoral bone loss following cementless THR. Our main endpoint was change in cortical bone. The actual measurement was made by calculating the cortical area (the area enclosed by the subperiosteal and endo-cortical bone surfaces) and the cortical porosity (the relative amount of cortical area occupied by pores larger in size than osteocytic lacunae). The cortical area and porosity of the operated limb were then compared to similar values for the contralateral, unoperated limb to determine the change in cortical bone.

1. Species Choice

The dog is considered the species of choice by many for studies of joint replacement because its bone microstructure is similar to humans, its remodeling kinetics have been extensively studied, the geometry of the proximal femur and mechanical properties of the bone of the distal femur and proximal tibia have been well-characterized, and its size is appropriate for studies of THR and TKR.^{54–58} Certainly, other species, most notably sheep and goats, but also pigs and rats have been used, but these models tend to be less well characterized.^{59–64} Perhaps, another advantage of the canine model, for THR, at least, is the fact that THRs are performed clinically in the canine.^{65–68}

2. Use of the Intact Contralateral Femur as a Control

From a methodological viewpoint, the desire to estimate the change in cortical bone raises certain issues. First, is it appropriate to use the contralateral limb as an intact control? We addressed this by examining the symmetry in geometric properties of the proximal canine femur, finding only very small side-to-side differences.⁶⁹ Although it is possible to monitor some aspects of limb function directly,^{70,71} we examine the bone mineral content of both tibiae either just at the end or during the entire course of the experiment, based on the assumption that these bone measurements are an adequate reflection of the loads placed on the limb integrated over time. Differences in limb function between the operated and control limbs or among animals in different groups could have a significant confounding effect on the primary endpoints. Previously, we have shown that tibial bone mineral content is symmetrical and that side-to-side differences are likely to be related to clinical function.⁷² In addition, another previous study showed that change in tibial bone mineral content of the

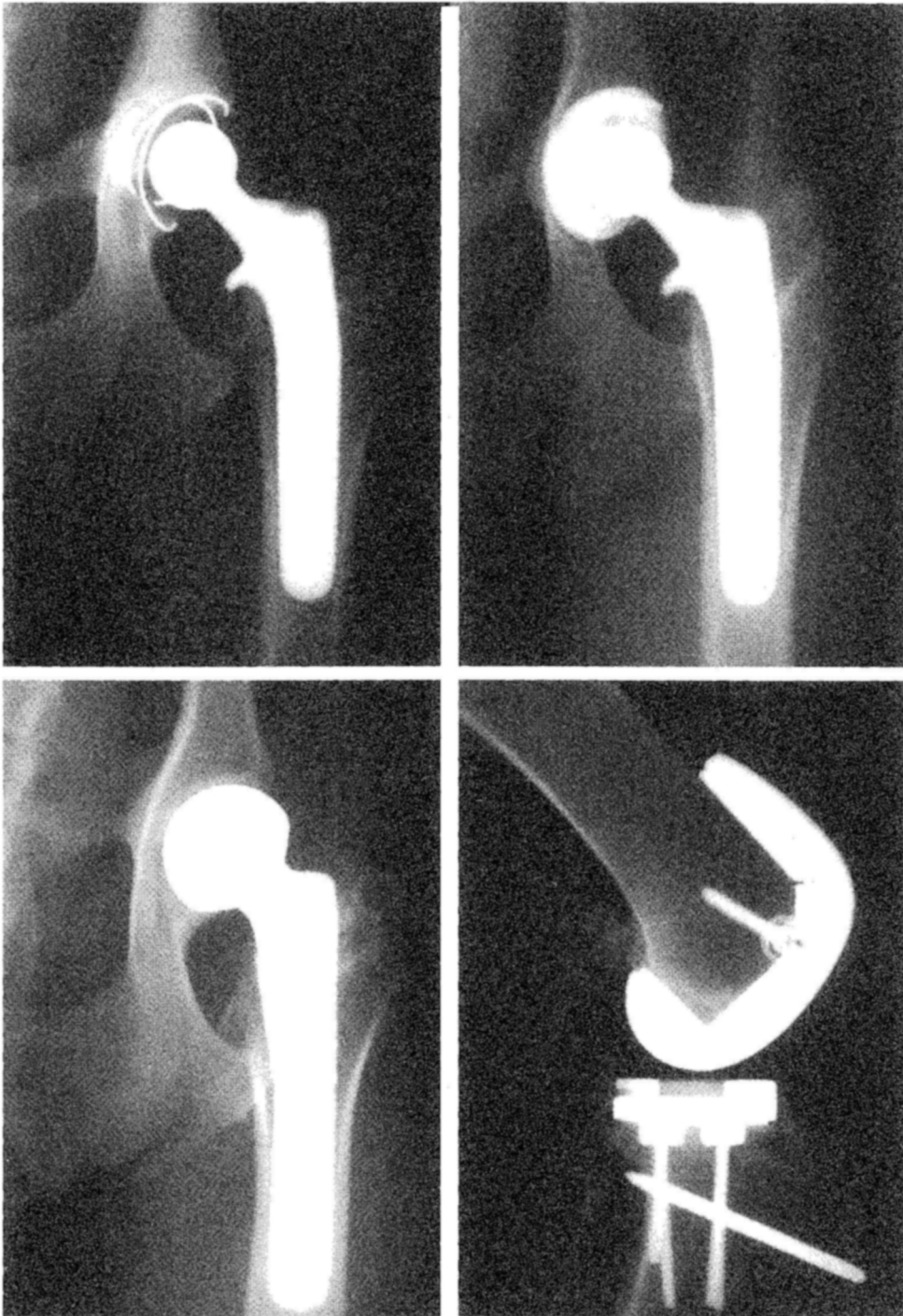


FIGURE 4. *In vivo* radiographs of three canine hip replacement models and one canine knee replacement model used by the authors. (a) a total hip replacement in which a cemented acetabular component is matched with a cementless femoral component, (b) a total hip replacement in which both the acetabular and femoral components are cementless, (c) a hemiarthroplasty in which only a femoral component is used with the head matched to the size of the intact acetabulum, and (d) a cementless knee replacement model with cementless femoral and tibial components.

contralateral femur can occur transiently but is minimal by six months.⁷³ All of these studies indicate that the contralateral limb is an adequate control. Given sufficient resources, an excellent additional control would be dogs that have not undergone surgery.

3. Sample Size

The next question is what should the sample size be? The answer is not simple, but depends on (1) the magnitude of the effect which the researcher believes is important, (2) the desired probability of showing a significant difference of this magnitude (i.e., the “power” of the significance test), (3) the significance level (usually 0.05) and (4) the variability in the data.^{69,74,75} The required sample size is inversely proportional to (1) and directly proportional to (2), (3) and (4). Thus, if the researcher wants a high probability of finding a small difference between two groups for an endpoint that is quite variable, a very large sample size will be needed. More specifically, if the researcher believes a 20% difference between groups is important and plans to use five animals per group, the research design (sample size) would be judged inadequate if one could only reasonably expect to demonstrate a 20% effect with 10 animals per group because of the expected variability in the data.

4. Time Points to be Examined

Another important question is what time points should be investigated? Of course there is no universal answer. However, if one is interested in long-term functional adaptation of bone it is important to choose a time frame which should allow several remodeling cycles to be completed. In the canine, the “activation-resorption-filling” sequence requires approximately two to three months. Interestingly, most studies have shown that most of the adaptation occurs within six months with minor changes occurring thereafter, but it is probably not correct to say that a new steady-state is reached already at six months. In addition, in some circumstances, e.g., if the implant is not fixed to the bone, there can be quite dramatic changes after six months.⁷⁶ Our personal opinion is that for functional adaptation studies, six months is a minimum and an additional time point is of value (such as one, two or even more years). To study transient events, such as the process of bone ingrowth, then shorter time points are of interest. We have focused on one month as a “screening” period, but other time points would be needed, depending upon the specific questions to be asked.

5. Experimental Endpoints

A critical issue is what should be observed and measured? Obviously, this depends upon the research question, but it is more involved because there may be unanticipated confounding variables. On the one hand, the researcher cannot possibly measure everything, but on the other hand, it can be devastating to miss something important. This problem is particularly vexing with joint replacement experiments because these experiments are inherently time-consuming and expensive. While one might be able to repeat experiments to make additional observations with rats, this rarely is the case with canines.

B. BRIEF REVIEW OF USAGE OF JOINT REPLACEMENT MODELS

Our review of the THR literature indicates that most of the attention has been on morphological and biomechanical responses with an emphasis on one or more of three primary regions: (1) the interface, (2) the adjacent tissue (usually medullary contents or trabecular bone) or (3) the host cortical bone (Table 1). We have used these two axes (type of question v. type of endpoint) to place a large body of THR studies in context (Table 1). While this classification scheme works for most studies, in some the focus has been elsewhere, such as the articular cartilage after hemiarthroplasty.^{77–80} A few studies have focused on biomaterial performance with little or no attention paid to the biological response. Some of these later studies are also listed in Table 1.

TABLE 1
List of *In Vivo* Hip Replacement Models, Sorted According to Type of Question and Type of Endpoint. The Last Part Lists Models in which the Primary Endpoint was Performance of the Biomaterial

Type of endpoint	Type of question		
	Interface	Medullary/trabec. bone	Cortical bone
Morphology	14,18-24, 40, 42-45, 53, 62, 66, 76, 81, 82 ,84 , 85, 90-104, 104-127	18-24, 53, 81, 82, 84, 92, 103, 128	22, 23, 44, 51, 53, 66, 76, 81-86, 98, 100, 101, 104, 105, 118, 119, 126, 128-133
Mechanical	24, 39-45, 76, 94, 116, 126, 134-136	137	44, 76, 76, 118, 119, 131, 132, 135, 137-139
Biomaterial performance	34, 77-80, 96, 105, 105, 140, 140-143		

Here, we review the THR models which have focused on morphological endpoints of the cortical bone to serve as a convenient example of the utility of Table 1. We summarize in Table 2 studies of functional adaptation of cortical bone in hip replacement models, basing our review on the appropriate cell of Table 1. Early work showed that the type of porous coating (as long as it allowed bone ingrowth) did not influence the cortical bone response.^{81,82} It was also found that the presence of a porous coating caused more bone loss initially, but eventually, animals with uncoated stems had as much bone loss as those with porous coated stems.⁷⁶ Work from Bobyn’s group suggested that use of proximally porous coated stems caused less cortical bone loss adjacent to the mid-stem than the use of fully coated stems,⁸³ but the experiment had a small sample size and, hence, low power. Comparison of two of our experiments suggests that even subtle differences in porous coating placement may influence the cortical bone response.^{81,84} Specifically, at six months we observed a tendency toward less bone loss with a stem that had the porous coating on the anterior and posterior faces only as opposed to one that had the anterior and posterior porous coating plus a small proximal medial area of porous coating. By far, the most consistent finding has been that cortical bone loss can be reduced by reducing the stiffness of the stem.^{53,84-86}

Knee models are much less common and it is not necessary to summarize the studies in tabular format. One of the early studies used a knee model to characterize the mechanical properties of fibrous tissue at the bone-cement-bone interface.²⁶ Later studies used these models to investigate various interface phenomena, including the potential to establish bone ingrowth fixation.^{25,27-29} More recently, knee models have been used to investigate the role of implant design on bone ingrowth and mechanical stability^{30,31,87} and wear of articular cartilage.⁸⁸

IV. CONCLUSION

We hope this chapter has shown that there is no perfect model because model choice depends upon the research question. In general, nonweight-bearing models are most useful for studies related to the establishment of implant fixation. Weight-bearing models are useful for issues related to establishment and maintenance of implant fixation. Thus, weight-bearing models are generally utilized for short and long-term studies (weeks to years) while nonweight-bearing models are used in short-term studies (weeks to months).

Since changes in the bony environment develop over time, conditions at the bone-implant interface (e.g., the development of fibrous tissue and migration of particulate debris or interface micromotion) or adjacent to the implant (e.g., loss of cortical bone) must be evaluated over long

TABLE 2
Summary of Femoral Cortical Bone Loss in Canine Models of Cementless Hip Replacement in which Unilateral Implants were in Place for Six Months or Longer

Length of femoral component	Porous coating location ¹	Material ²	Length of study (months)	Total sample size	Proximal cortical bone loss ³	Mid-stem cortical bone loss ³	Reference
73 mm	80%, A+P	Ti alloy	6	9	11%	10%	84
73 mm	80%, A+P	Composite	6	9	4%	6%	84
88 mm	94%, circumferential	Ti alloy	6, 24	22	35%	20%	53
88 mm	94%, circumferential	Composite	6, 24	22	20%	10%	53
73 mm	80%, A+P+prox M	Ti alloy	6, 24	32	15%	15%	81,82
73 mm	80%, circumferential	Ti alloy	6, 24	10	25%	15%	81,82
75 mm	100%, circumferential	CoCr	16, 36	4	20%	25%	83
75 mm	40%, circumferential	CoCr	16, 36	4	25%	2%	83
?	67%, circumferential	CoCr	6, 24	10	—	12%	130

1. Porous coating location; the percent refers to the distal extension of the porous coating, A = anterior stem face, P = posterior stem face, M = medial stem face.
2. Material: Ti = titanium; composite = a relatively low stiffness implant; CoCr = cobalt chrome.
3. Cortical bone loss = difference in cortical area between the operated femur and the contralateral (control) femur. When two time points were available the means or individual data points were averaged because, in general, none of these studies showed a difference in bone loss as a function of time. These are mean values. Some individual cases showed considerably more and some considerably less bone loss.

periods of time. It is apparent that clinically relevant issues, such as adaptive remodeling^{52,89} or the changes that occur at the bone implant interface³⁴ can be well-studied experimentally in weight-bearing models.^{14,53,90} The ability to study these issues in animal models allows relatively rapid accumulation of information that otherwise would take years to acquire from human autopsy material.

ACKNOWLEDGEMENT

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22 Animal Models for Investigations of Biomaterial Debris

Martin Lind, Yong Song, and Stuart B. Goodman

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I. INTRODUCTION

Total joint replacement (TJR) is a very successful procedure for the treatment of end stage arthritis. However, TJR still has problems with regards to wear and late loosening of the components. These problems are becoming more important since the indications for TJR have been extended to a younger patient group. Also the elderly population is expecting an extended and more active lifetime and both of these situations will place increasing demands on TJR both with respect to survival-time and durability.

A key factor for the loosening of prosthetic components is believed to be the generation of and the biologic response to wear particles in periprosthetic tissues. Wear debris typically is generated

at the normal articular surfaces of a TJR. A person can perform over 1 million gait cycles in a year and this activity can potentially generate hundreds of millions of wear particles from the metal and polyethylene (PE) articulating surfaces.²³ Particles can also be generated from the interfaces of modular components such as metal backed PE components. The generation of wear debris can be accelerated if third body wear (bone, metallic or other debris) is trapped in the articular surface or if abnormally articulating interfaces exist (such as areas of impingement between the femoral neck and the acetabular cup). The generated particles are typically small ($<10\text{ }\mu\text{m}$) and, therefore, are phagocytosable by macrophages.²³ The macrophages that participate in the phagocytosis become activated which leads to secretion of numerous substances that interact with fibroblasts, osteoblasts, osteoclasts and also other cells from the immunological system.^{15,22} This biological reaction modulates the formation and resorption of mesenchymal tissue and eventually leads to some pathological findings in failed TJRs including membrane formation, perimplant osteolysis, and implant loosening.^{33,34}

Improved understanding of the biological response to wear debris from TJR is important for revealing the mechanisms of failure of prosthetic components and for possible future interventions for prevention and treatment. Animal models have contributed greatly to the understanding of the biological processes that are involved in the response to wear debris particles in soft and hard tissue.

Animal studies are valuable for a number of reasons. It is possible to develop standardized models in which a particular facet of a complex biological process can be investigated. Particles of known size and material can be implanted at specific locations and at controlled doses. Biological specimens can be harvested at any time point and end point determinations are almost unlimited. Animal models also have the advantage of investigating biological effects in an intact organism where the complex interactions between different tissues are preserved. This is contrary to *in vitro* studies in which single cell cultures lack interactions from other cell-types and extracellular matrix. The disadvantage of animal experiments is the high expense of such studies. Also, it can be difficult to select a model that mimics exactly the surgical situation or biological process in humans that is of interest. Another problem is that different physiology in animals, especially lower species, can cause difficulty in extrapolating data from animal experiments to human situations. This is especially relevant for bone physiology, in which bone remodeling in rodents is very different from humans and healing is faster in animals than in humans.

Despite these disadvantages animal studies have contributed to the understanding of the biological reaction to orthopaedic wear debris. It is the scope of this chapter to review the different animal models that have contributed to the understanding of the biological effects of wear debris and to summarize the data retrieved in these studies. We have chosen to divide the models into two major groups: first models with application of wear debris alone into different tissues, and second models in which wear debris is applied in the interface between bone and an implant.

II. MODELS WITH PARTICLES WITHOUT IMPLANTS

A. INTRAMUSCULAR AND INTRAARTICULAR INJECTION OF PARTICLES²⁹

In this early study by Stinson, particles were injected into the left gluteal muscle and left knee joint of Hartley guinea pigs. Particles of polymethylmethacrylate (PMMA), PE and nylon with a size range from 0–72 μm were investigated. Animals were sacrificed at two weeks, 1, 6, 12, 24, 30 and 36 months after implantation of particles.

Muscle biopsies were analyzed by qualitative histology and knee joints were investigated by macroscopic evaluation of particle location and joint cartilage status as well as qualitative histology of synovial tissue where particle accumulation was observed.

In muscle tissue, necrotic muscle fibers were seen in relation to particles, which evoked a macrophagic reaction. At later time points up to 12 months, necrosis disappeared and foreign body

giant cells predominated. Foci of plasma cells and lymphocytes were very evident at 12 months, typically located adjacent to clumps of particulate material. Giant cells were replaced by fibroblasts at late time points from 12 month for PE particles. Nylon particles were removed and not seen after 18 months. In knee joints, particulate PMMA was accumulated in synovial tissue as early as after two weeks. At two and four weeks the particles were primarily surrounded by macrophages. At later time points particles were surrounded by a fibrous stroma with islands of foreign body giant cells. Small foci of plasma cells and lymphocytes were seen at 18 and 24 months. The joint cartilage was unaffected by particle implantation at all time points. PE and nylon particles gave similar synovial reactions.

B. SUBCUTANEOUS POCKET MODEL

CD rats (four weeks old, Charles River Breeding Labs., MA)⁶ and MF1 mice (six weeks old)²⁵ were used for this model. Bilateral subcutaneous pockets were prepared by blunt dissection over the thoracic area. In the study by Glowacki et al. 50 mg of PMMA, polyethylene and bone particles were placed in the pockets for 12 days.⁶ The particle size ranged from 75–250 μm . In the study by Quinn et al.²⁵ PMMA particles were placed in the pockets for two months. The particle size ranged from 50–300 μm .

Glowacki et al.⁶ used qualitative undecalcified histology and semiquantitative histochemistry to evaluate inflammatory and osteoclast response to the different particles. Quinn et al. harvested the granulomas formed at the particle application sites. One part of the granuloma was used for histology and another part was used for cell culture. The granuloma cells were then co-cultured with fibroblastic, osteoblastic and stromal cells on bone slices for evaluation of osteoclastic activity.

Glowacki et al.⁶ found that these nonphagocytosable PMMA and PE particles were surrounded by foreign body giant cells in fibrous stroma. The bone particles were surrounded by osteoclasts that showed evidence of surface resorption. Histochemical analyses for an osteoclast marker confirmed a high osteoclastic activity around bone particles. Quinn et al.²⁵ found mononuclear inflammation with scattered giant cells in response to PMMA particle implantation. These cells stained positive for macrophage markers but not for osteoclast associated tartrate resistant acid phosphatase. The granuloma cells exhibited osteoclastic activity on bone slices after 14 and 21 days of co-culture with osteoblastic or stromal cells but not after co-culture with fibroblastic cells.

C. SUBCUTANEOUS AIR POUCH MODEL²¹

Under anesthesia 20 ml of air was injected into the subcutaneous tissue of a 6–8 week-old SD rat's back to form a single subcutaneous air pouch. At day 5, an additional 10 ml of air was injected together with 5 ml of particle suspension. In this study PMMA particles (11 μm diam.) with and without 10% barium sulfate were injected. Liquid from the air pouch was aspirated at 1, 6, 12, 24, 72 hours and 1 and 2 weeks by injecting 5 ml saline and aspirating 4 ml for analyses.

The air pouch aspirate was analyzed for leukocyte count. Prostaglandin E_2 (PGE_2) levels were measured by enzyme linked immunosorbent assay (ELISA). Tumor necrosis factor (TNF α) was measured by L929 bioassay, and metalloprotease activity was measured by a substrate degradation spectrophotometric assay.

Particulate PMMA with barium sulfate was associated with an earlier influx of leukocytes than PMMA particles without barium sulfate at six hours. At the same time point, increased metalloprotease activity was demonstrated for PMMA particles with barium sulfate. PGE_2 release was highest in the barium sulfate group from 1–24 hours whereas TNF α was increased at 48 and 72 hours. It was concluded that addition of barium sulfate to bone cement for radioopacity could result in an accentuated host inflammatory response to particulate debris from the cement.

D. RABBIT TIBIA MODEL^{10-12,14}

Mature NZW rabbits (3-4 kg) were used for this model. After bilateral exposure of the anteromedial aspects of the proximal tibia a 6 mm drill hole was made by a hand drill. A curved curette was used to scoop bone marrow out of the canal underneath the drill hole. After saline irrigation and drying the hole was implanted with standardized amounts of either bulk or particulate material in the right tibia; the left tibia served as a non-implanted sham operated control. Particles of ultrahigh molecular weight polyethylene (UHMWPE), Ti6Al4V alloy, cobalt chromium alloy (CoCr alloy), and PMMA were analyzed. Particle sizes were <1000, 4, 15 and 10-100 µm respectively. Observation time was 16 weeks, but harvesting can occur at any time period(s).

Sterile harvest of bone marrow was used for organ cultures. Weight standardized tissue samples were cultured in Dulbecco modified Eagle's medium for three days and conditioned medium was assayed for PGE₂ levels by radioimmunoassay (RIA). Also, the proximal tibia was harvested and prepared for decalcified histology by conventional techniques. The bone implant interface was analyzed by counting giant cells, histiocytes, lymphocytes, plasma cells, PMN leukocytes, fibroblasts and marrow cells using a standardized system.

Particulate PMMA material exhibited 2.25 fold higher levels of PGE₂ in bone marrow organ cultures compared to controls. Bulk PMMA exhibited identical PGE₂ levels as controls. This response could be inhibited by giving the animals an oral nonsteroidal anti-inflammatory drug, sodium naproxen, during the observation period. Using histological analyses, particulate UHMWPE and PMMA implantation resulted in a bone implant interface with increased cell counts for giant cells and histiocytes when compared to bulk implantations of the same materials. Similar implantation of particulate and bulk titanium (Ti) and CoCr alloy did not demonstrate any increase in giant cells and histiocytes in the particulate material group.

E. THE BONE HARVEST CHAMBER WITH PARTICLES^{1,7,9,32}

The bone harvest chamber (Figure (1)) is a Ti device that is implanted in the proximal tibial metaphyses of mature male NZW rabbits. The chamber has a 1x1x10 mm pore for tissue ingrowth at the cortical bone level. The top of the chamber can be accessed through a small skin incision and be disassembled for tissue harvest or particle application. In these studies chambers were inserted bilaterally. After osseointegration, repeated harvests of tissue growing into the chamber are possible. The particles investigated were machined high density polyethylene (HDPE), hydroxyapatite (HA), Ti6Al4V alloy and CoCr alloy particles with 4.7, 5.0, 3.0 and 2.7 µm mean diameter respectively. Also diamond and SiC particles with a <20 µm size were investigated. Particles were dissolved in sodium hyaluronate at a concentration of 1x10⁸ particles/ml. Initially a six week osseointegration period was applied. Then the carrier material (Healon) was placed bilaterally for three weeks. Tissues were harvested and HDPE particles were placed in the chambers on one side and the contralateral side was left empty as control. After another three weeks tissues were harvested. The previous control side now received titanium alloy (Ti alloy) particles and the contralateral side served as control for a final three weeks.

The tissue specimens harvested were prepared for conventional decalcified histology. Amount of bone and fibrous tissue was quantified as well as cellular parameters of foreign body reaction (histiocytes and giant cells), acute and chronic inflammation (polymorphonuclear leukocytes and lymphocytes/plasma cells).

CoCr alloy and HDPE particles caused 67% and 35% reduction in bone ingrowth into the chamber whereas Ti alloy particles did not exhibit this effect. Also in CoCr alloy and HDPE treated tissue specimens particles were surrounded by mono- and multinucleated histiocytic cells as well as lymphocytes and plasma cells in a fibrous stroma. In tissue specimens receiving Ti alloy particles the cellular composition was similar to control tissue specimens. HA particles increased bone

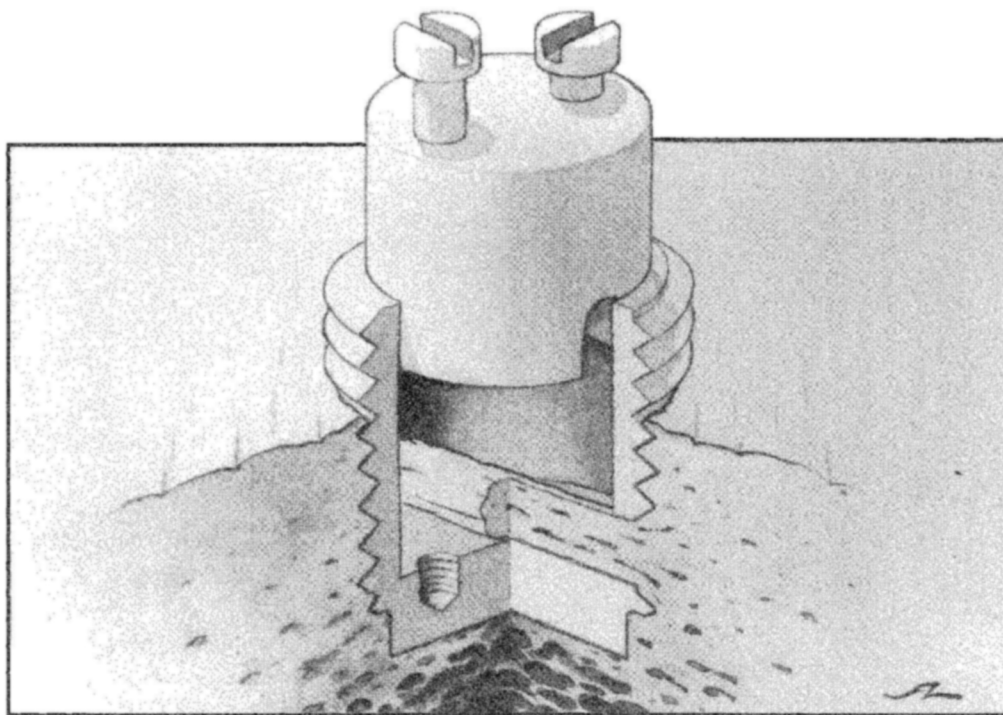


FIGURE 1. The bone harvest chamber. The canal for bone ingrowth at the cortical bone level is illustrated as well as the removable core that enables access to the bone canal for particle application of tissue harvest.

ingrowth at three weeks and no inflammation and granuloma formation was seen. Diamond and SiC particles had no adverse effects in the bone harvest chamber.

F. MODEL WITH PARTICLE APPLICATION TO MICE WITH DIFFERENT DEGREES OF IMMUNODEFICIENCIES^{19,20}

Four different strains: normal immunocompetent mice, nu/nu athymic mice deficient in T cells, severe combined immunodeficiency strain (SCID) mice with decreased T and B lymphocyte function, and triple deficient mice (NIH III, nude-beige) lacking T and B lymphocyte function as well as natural killer cells were used.

Using the immunocompetent and immunodeficient mice, Jasty et al.¹⁹ and Jiranek et al.²⁰ examined the tissue response to injection of PMMA powder. The PMMA particles were 13.6 μm in diameter and approximately 25,000 particles in 0.2 ml aliquots were injected into two ventral and two dorsal subcutaneous sites of each mouse strain. A tissue reaction was then allowed to form in five weeks.

Tissue granulomas formed were analyzed by immunohistochemistry for leukocyte markers: Mac-2 for macrophages, CD3 for T-lymphocytes, CD8 for antigen expressing suppressor T-cells and CD20 for B-lymphocytes. *In situ* hybridization for IL-1 β was applied to locate gene expression of this cytokine.

All animal strains demonstrated granulomas when PMMA powder was injected subcutaneously for five weeks. All four mouse strains exhibited the same macrophage accumulation unaffected by the different degrees of deficiencies in immunocompetence. The macrophages in the granuloma expressed messenger RNA for interleukin-1 β , a marker for macrophage activation, and the

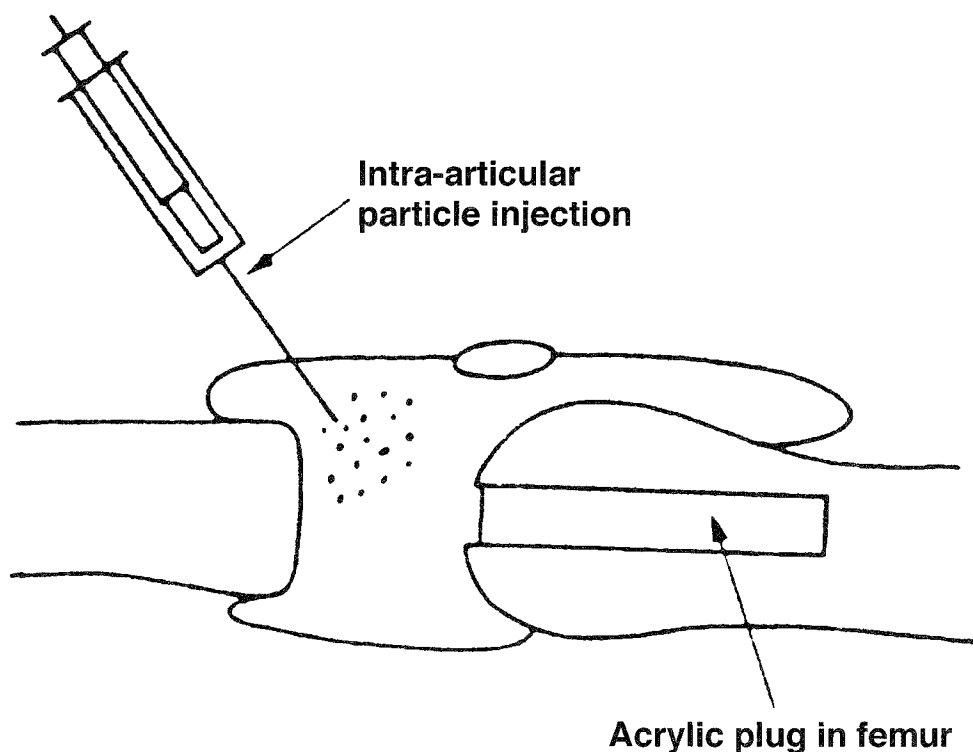


FIGURE 2. The Howie model. The rat knee joint is illustrated with the implanted bone cement plug in the distal femur. Also the site for particle injections is shown.

expression was independent of lymphocyte presence. It was concluded that macrophages are the key cells initiating and maintaining the foreign body response to particulate PMMA, and that lymphocytes are not essential to mounting this response. A similar finding was demonstrated in the rat tibia model using normal and T-cell deficient animals and HDPE particles.⁸

III. MODELS WITH PARTICLES AND IMPLANTS

A. RAT MODEL WITH INTRAARTICULAR PMMA PLUG AND APPLICATION OF PE PARTICLES

In this model by Howie et al.,¹⁸ through a medial knee arthrotomy, a hole (10 mm in length, 1.1 mm diam.) was drilled in the intercondylar notch of mature Lewis rats. A PMMA plug was then inserted into the drill hole so that the distal end of the plug was just below the level of the articular surface. (Figure 2) Post surgery the operated knees were injected with 1 mg UHMWPE particles (20–200 μ m diam.) dissolved in 15 ml of a 1:50 serum/saline solution. Injections were performed at two, four, six, and eight weeks postoperatively and the animals were terminated at 10 weeks. A control group received no injections and were terminated at two weeks.

The knee joint and distal femur were harvested for conventional decalcified histological preparation. The interface was investigated by qualitative histology.

In control animals, a complete shell of bone had formed around the implant and a very thin layer of amorphous tissue was located between the cement and bone. No signs of bone resorption were seen. In the animals injected with particles, the bone surrounding the implant had been replaced by a cellular connective tissue composed of macrophages, giant cells and fibroblasts. Bone resorption was

most evident close to the joint surface but osteoclasts were also seen on bone surfaces some distance from the joint. Throughout the tissue surrounding the implant, birefringent PE particles were demonstrated using polarized light. This study was the first to demonstrate formation of a peri-implant connective tissue membrane as a result of intraarticular particle exposure.

Using a modification of the Howie model, van der Vis et al.³¹ have recently tested numerous different particle types. In their study, the model was modified by placing the particles around the PMMA implant at surgery only and the observation time was four weeks only. The study confirmed that PE particles stimulate peri-implant bone resorption. CoCr alloy, Ti6Al4V alloy and zirconium oxide particles did not have this effect.

B. CANINE MODEL WITH DIFFERENT IMPLANT TEXTURES AND PE PARTICLES

In this model by Bobyn et al.³ special cylindrical implants were inserted intraarticularly in the knee joints of mature mongrel dogs. The implants had a smooth texture on half of the surface and a porous coating on the remaining surface. The implants were 9x30 mm and were press-fit in the intercondylar notch of the femur and in the tibial plateau bilaterally. At both locations, implantations were performed parallel to the long bone axis. Initially a 10 week osseointegration period was allowed before intraarticular injections with polyethylene particles began. Thereafter, the animals were injected twice weekly with machined (4.7 μm diam.) polyethylene particles for 10 weeks. Two doses were investigated, 5 and 10 mg per injection in the left and right knees respectively. The particle injection period was followed by another 10 week period before sacrifice. In this period an inflammatory response to the injected particles was allowed to evolve.

After sacrifice the distal femur, proximal tibia and synovial tissue were harvested for conventional undecalcified thin section histology and contact radiography. Four to eight sections at different levels from each implant were analyzed qualitatively for bone ingrowth and periprosthetic membrane formation and for the presence of PE particles within the bone/implant interface.

Synovium from particle-injected animals contained large amounts of PE particles. The smaller particles were seen intracellularly in macrophages and larger particles typically were surrounded by foreign body giant cells. For all implants that were subjected to particles, bone ingrowth was seen at the porous coated surface whereas the smooth surface was surrounded by peri-implant fibrous tissue. Corresponding radiolucent lines were observed at contact radiography in the latter group. The cavities around the smooth implants were observed from the most proximal to the most distal parts of the implant and were of fibrous character. The bone cavities around the smooth implant surfaces were surrounded by a distinct bony shell or neocortex and PE particles were found to have migrated almost exclusively along the smooth surface of implants. This model is the first to show that a porous coated implant surface can prevent particle migration into the bone/implant interface.

C. TOTAL HIP ARTHROPLASTY MODELS

Two models to investigate the effects of wear particles in a total hip arthroplasty model have been described by Spector et al. and Dowd et al.^{4,28}

1. Spector Model²⁸

Unilateral implantation of a Charnley like femoral stem and a PE acetabular cup was performed in seven dogs. Prior to implantation a thin layer of bone cement (1–2 mm) was applied to the femoral stem in the laboratory. During surgery the medullary canal was over reamed so that the cement coated stem could be inserted in a loose fit to enable motion of the stem. Before the stem was inserted 250 mg of bone cement particles (500 μm diam.) was placed in the femoral canal. It was hypothesized that abrasion of these bigger particles, between each other and the cement sheath on the stem, would generate smaller phagocytosable particles. Animals were sacrificed at four and seven months.

Evaluation was performed by anteriorposterior and lateral radiographs preoperatively, immediate postoperatively, two, four and seven months postoperatively. Radionucleotide imaging was performed one day prior to sacrifice by intravenous injection of ^{99m}Tc -methylene diphosphonate. The femur was excised and the membrane was removed aseptically. Part of the membrane was used for histology and embedded in methylmethacrylate. The other part of the membrane was used for biochemistry. This was accomplished by dicing the tissue and subsequent collagenase treatment to release the cells. Mononuclear cells were isolated by Ficoll gradient centrifugation and subsequently cultured for 72 hours with and without sodium naproxen. Conditioned media were assayed for PGE_2 and interleukin 1 (IL-1) content as well as bone resorbing activity in the fetal rat calvaria organ culture assay.

A radiolucent seam was found at the cement bone interface at four months; at seven months this seam became wider and more irregular. Subsidence of the stem was also seen. Radionucleotide imaging showed increased blood pooling and bone turnover around the operated stem when compared to the non-operated femora. However, no changes in blood flow were observed. Histologically the membrane tissues demonstrated multiple layers of macrophages in a fibrous stroma as well as multinucleated giant cells around PMMA particles. PGE_2 and IL-1 production from the cultured membrane cells could be depressed by naproxen treatment. The medium from the treatment group stimulated bone resorption more than control medium.

2. Dowd Model⁴

Thirty eight dogs received unilateral implantation of cementless femoral stems with a midstem porous coating and porous coated acetabular cup. Three different prosthesis types were used: (1) A control prosthesis with a cementless implant design. (2) A gap prosthesis with a midshaft circumferential 2 mm gap in which different particles could be applied. (3) A motion prosthesis with a midshaft ball joint to enable implant motion. The dogs were randomized to six different groups including, (1) control prosthesis, (2) motion prosthesis, (3) gap prosthesis without particles, (4) with CoCr alloy particles, (5) with Ti6Al4V alloy particles, and (6) with HDPE particles; 100 mg of particles were placed in the gap. The particle sizes ranged from 3–10 μm in diameter. The observation period was 12 weeks.

After sacrifice the proximal femur with implant was harvested as well as synovial tissue from the contralateral hip joint. Part of the membrane was used for histology and graded semiquantitatively with respect to macrophage density. Another part of the membrane was harvested aseptically and used for biochemistry. This was accomplished by dicing the membrane and synovial tissue and subsequently culturing the tissue in organ culture for 72 hours. Conditioned media were assayed for collagenase, gelatinase, PGE_2 and IL-1 activity.

Manual testing demonstrated that the control implants were well-fixed after the 12 week observation period whereas the implants with motion or particles were all manually loose. Histologically, membrane tissue around control implants consisted of very few macrophages. For motion implants a thick membrane was found but with only a moderate number of macrophages. Around the gap implants, a thick membrane was found with high numbers of macrophages in all particle stimulated groups. However, PE particles appeared to stimulate macrophage accumulation less than CoCr alloy and Ti alloy particles. Biochemical analyses of conditioned medium from cultured membrane tissue revealed high levels of collagenase activity and PGE_2 release in metal particle groups and lesser activity in the polyethylene particles group. Gelatinase activity was mainly elevated in the group with motion implants. IL-1 activity was highest in CoCr alloy and PE particle groups.

D. RABBIT TIBIAL HEMIARTHROPLASTY WITH FIXED AND LOOSE IMPLANT AND PMMA PARTICLES^{13,16,17}

Using a medial parapatellar incision, the right knee joints of adult NZW rabbits were accessed. Anterior cruciate ligament and menisci were removed and the tibia plateau cartilage and a small

amount of bone were removed by an oscillating saw. Eight animals received fixed implants by conventionally cementing a custom type stemmed and fluted titanium alloy tibial hemiarthroplasty implant in place. Another eight animals received a loose implant. This was accomplished by letting the cement cure *ex vivo* after its application to the implant. When placed in the prepared tibial plateau an additional thin layer of PMMA powder (10–100 μm particles) was applied to the surface of the implant/cement complex to simulate cement debris. To ensure loose fitting, the implant was rotated 90 degrees once. Observation time was three months.

The bone/implant interface was investigated by histology and radiography. Sterile harvest of bone marrow from the proximal 2 cm of the tibia was used for organ cultures. Biopsies from both operated and non-operated tibia were harvested. Weight standardized tissue samples were cultured in Dulbecco modified Eagle's medium for three days and conditioned medium was assayed for PGE₂ levels by RIA and lysosomal enzyme activity.

Wider radiolucent lines and a thick fibrous membrane were observed around the loose implants. Bone marrow from the particle exposed loose implant group produced three times more PGE₂ than non operated marrow. In the fixed group PGE₂ production was less than in non operated marrow. Also lysosomal enzyme activity was increased in the loose implant group.

E. RAT MODEL WITH COMBINED PARTICLE APPLICATION AND MICROMOTION

Male SD rats (350 gm) were used in this model.² A 4×13 mm Ti plate was screwed onto the medial surface of the proximal tibia. In the middle of the plate there was a hole into which a circular 2.5 mm test surface could be screwed to contact the cortical bone surface. The test surface protruded 0.5 mm into the cortical bone which was milled at the area to receive the test surface and also to induce bone trauma and hematoma. HDPE particles (machined to 4.7 μm) were applied initially or at later stages by unscrewing the test surface. Implant movement was applied by rotating the test surface 180 degrees 20 times twice daily. Movement was achieved by manually rotating a subcutaneous located wing nut on the test surface by external manipulation. Several investigational groups were included: (1) the cortical bone was allowed to osseointegrate around the test surface for six weeks and then particles were applied for six weeks or particles for six weeks only without any prior osseointegration. (2) osseointegration for two weeks and then motion for two, or six weeks³ osseointegration for two weeks and then motion and particles for six weeks, or motion for six weeks followed by particles for six weeks.

Histomorphometry was performed on conventional decalcified sections to quantify bone and cartilage fractions at 0.18 and 0.45 mm below the test implant surface. Also the thickness of a soft tissue membrane between implant and bone was measured.

Application of particles alone did not disturb osseointegration and this result was similar for implants with particles from the implantation time or after six weeks osseointegration. Movement created a fibrous membrane that was well established at six weeks and contained small islands of cartilage. When both particles and motion were present, the membrane was similar to the membrane seen with motion alone without signs of inflammation. If particles were applied after six weeks of movement, osseointegration that was otherwise seen in animals rested six weeks after micromotion was prevented.

IV. MODELS WITH INTRAARTICULAR PARTICLE INJECTIONS AND JOINT IMPLANTS

A. RABBIT MODEL

Mature NZW rabbits (3.0–3.5 kg) were used in this model.⁵ Through a medial parapatellar incision the knee joint was accessed for intercondylar implantation of a Ti alloy implant (5 × 10 mm)

bilaterally. Observation time was 12 weeks. During this period weekly intraarticular injections of 1 mg HDPE particles (7 μm) suspended in hyaluronic acid were performed. Particles were injected on one side and sham injection was performed on the contralateral side.

Synovial tissues were harvested for semiquantitative determinations of particle infiltration and immunohistochemical staining for the macrophage chemoattractant cytokines interleukin 8 (IL-8) and macrophage chemotactic and activating peptide 1 (MCP-1).

In control joints, no signs of inflammation and staining for IL-8 and MCP-1 were found. In joints receiving particles, moderate inflammation with mainly mononuclear cells were seen. Particles were seen beneath the lining layer of the synovial membrane. The synovial tissue demonstrated intensive staining for MCP-1, but moderate IL-8 staining.

B. CANINE MODEL

Using mature Labrador dogs, Ti alloy implants with and without HA coating were inserted bilaterally into the weight bearing part of the femoral condyles surrounded by a 0.75 mm gap.²⁶ The implants were mounted on a special weight bearing device that ensured implant loading during each gait cycle and also created access to the joint space.³⁰ Each femur received one uncoated and one HA coated implant. One knee joint was randomly selected for particle injection of 6×10^9 HDPE (2 μm) particles. The contralateral knee had implants inserted but received no particles. Particles were injected weekly from week three and throughout the eight-week observation period.

The implant bone specimens were investigated histologically using decalcified sections for analysis of particle migration along the bone implant interface and undecalcified sections for analysis of bone and fibrous tissue ingrowth.

Particles were found to have migrated along the uncoated implants in large numbers, whereas almost no particles were found around the HA coated implants. Less bone ingrowth and bone formation in the gap along with more fibrous tissue formation were found around uncoated implants when compared with HA coated implants.

V. CONCLUSION

In vivo studies have contributed greatly to the understanding of biological effects of orthopaedic wear debris (Tables 1 and 2). Models without implants used various locations for particle application such as muscular, subcutaneous, bone marrow and periimplant tissue. Particles applied in these locations were able to generate granuloma formation rich in macrophages and giant cells. With longer observation time, from 12 months and longer lymphocyte aggregates typically were seen in these granulomas.^{6,25,29} Of the many materials used in orthopaedic implants such as: PE, Ti6Al4V alloy, CoCr alloy and PMMA, PE seems to form the most aggressive granuloma.³¹ Particles have to be of phagocytosable size ($<20 \mu\text{m}$) to elicit an inflammatory response with granuloma formation.^{11,12,14} When particles are applied around implants inserted in bone, fibrous tissue membrane formation and bone resorption have been demonstrated in many models.^{4,18,28} Some studies suggest that polyethylene causes less accumulation of macrophages around implants than CoCr and Ti6Al4V alloy particles, but which particle material generates the most aggressive granuloma formation seems to depend on the model used.⁴ It appears that motion at the bone implant interface produces a more fibrous membrane than particles alone. This membrane is, however, without many macrophages that are seen in particle associated membranes. Implants with a smooth surface texture seem to give access to more particle accumulation at the bone-implant interface than implants with a porous coating or a HA coating.^{3,26}

In summary, animal studies have confirmed that application of orthopaedic wear debris particles can lead to radiographically evident osteolysis and formation of macrophage rich fibrous tissue membrane. Furthermore, particle induced granuloma express mediators that participate in accelerated bone resorption such as IL-1b, IL-6, and PGE₂. These characteristics all mimic the pathological

TABLE 1
Animal Models for Effects of Wear Debris Particles (Without Implants)

1st author year ^{Ref.}	Species	Model, Observation time	Particle type/size	End point
Stinson 1965 ²⁹	Rat	IM and intraarticular particle injections, days-2 years	PMMA, PE, Nylon, <70µm injections every 2nd week	Qualitat. histology
Glowaski 1986 ⁶	Rat	Subcutaneous pockets, 12 days	PMMA, 75-250 µm	Qualitat. histology, Semiquantitative histochemistry
Goodman 1992 ^{10,14}	Rabbit	Rabbit tibia hole with particles, 16 weeks	PMMA, PE, Ti6Al4V, CoCr 10-100 µm	Organ culture marrow from prox. tibia, PGE2 measurements, Quantitat. histology
Quinn 1992 ²⁵	Mouse	Subcutaneous pockets 2 months	PMMA, Bone, PE, 50-300 µm	Qualitat. histology, Co-culture bone lice osteoclast assay, IL-1 synthesis.
Goodman 1992 ^{7,9}	Rabbit	Bone harvest chamber 3 weeks	PE, Ti6Al4V, CoCr, HA, SiC, Diamond, 2-20 µm	Bone ingrowth, Quantitative histology
Jasty 1992, ¹⁹ Jiranek 1995 ²⁰	Mouse	SC particles in immunodeficient mice, five weeks	PMMA, 13.6 µm	Immunological cell counts in granuloma
Lazarus 1994 ²¹	Rat	Air pouch model one hr.-2 weeks	PMMA, 11 µm +/- BaSO4	TNF-α, PGE2 metallo-proteinase in pouch liquid

TABLE 2
Animal Models for Effects of Wear Debris Particles (With Implants)

1st author year ^{ref.}	Animal	Model, Observation time	Particle type/size, use	End point
Howie 1988 ¹⁸	Rat	Intraarticular PMMA rods in knee joint, 10 weeks	PE 20–200 µm, injections every 2nd week	Qualitative histology
Spector 1990 ²⁸	Dog	Total hip arthroplasty 4–7 months	PMMA, 500 µm	Qualitative histology and radiography
Goodman 1992 ^{16,17}	Rabbit	Total knee arthroplasty cemented fixed and loose, 3 months	PMMA, 10–100 µm	Organ culture marrow tissue from proximal tibia, PGE2 measurements
Dowd 1995 ⁴	Dog	Total hip arthroplasty, 12 weeks	CoCr, Ti6Al4V, PE, 4–10 µm	Quantitative histology, enzymes, PGE2 and IL-1 synthesis
Frøkjær 1995 ⁵	Rabbit	Intraarticular titanium implants in knee joint, 12 weeks	PE, 7 µm, weekly injections	Immunohistochemistry of synovial tissue for IL-8 and MCP-1
Boby 1995 ³	Dog	Titanium implant in knee joint, half smooth, half porous, 30 weeks	PE, 4.7 µm twice weekly in middle 10 weeks	Qualitative histology
Aspenberg 1996 ²	Rat	Tibial implant with micromotion + particles, six weeks	PE, 4.7 µm	Quantitative histology
Rahbek 1997 ²⁶	Dog	Weight-loaded implant with gap in knee joint, 8 weeks	PE, 2 µm, weekly injections	Mechanical tests and quantitative histology

scenario observed clinically in aseptic loosening of prosthetic implants. This large body of experimental evidence points towards a significant role for wear particles in the etiology of aseptic loosening. Further research in this field could lead to future interventional steps that could improve the clinical outcome of prosthetic surgery.

VI. FUTURE RESEARCH DIRECTIONS

Currently the histopathological effects of orthopaedic wear debris particles have been investigated and described in detail. Many of the inflammatory mediators that are expressed and/or regulated during particle induced inflammation have been identified. However, there are still many poorly understood facets of particle induced inflammation and its contribution to prosthetic implant loosening. The clinical picture in patients having a prosthetic implant with known wear debris problems varies. Some patients have well functioning prostheses and are symptom free for decades despite extensive PE wear and particle generation. Other patients develop extensive osteolysis and implant failure within few years. Little is known about this difference in biological response to prosthetic component implantation and the individual response of patients to wear debris particles. Also the possible involvement of the immune system in the reaction to particulate wear has been implicated. Several studies with particle implantation for 12 months and longer have observed lymphocyte accumulation in the particle generated granuloma tissue.

Immunohistochemical studies have demonstrated that some of these lymphocytes express the IL-2 receptor, which is a marker of lymphocyte activation. However, no studies have shown specific lymphocyte reaction or specific antibody production in response to wear particles. New animal models that can detect such lymphocytic activation in long-term studies are needed to answer the question of immune system involvement.

Another important direction for future research is to develop methods and techniques that can prevent the adverse effects of particle induced inflammation in periprosthetic tissues. Such approaches could target the implants, and recent studies have shown that a porous coating or HA coating on a cementless implant can prevent some of the particle migration along the implant-bone interface.^{3,26} Another recent study has used a pharmacological approach for treatment of aseptic loosening of prosthetic components. In this study by Shanbhag et al., total hip arthroplasties were performed in dogs. If a bisphosphonate alendronate, was administered, osteolysis could be prevented despite the fact that periimplant membranes still produced the high levels of cytokines.²⁷

Finally, recent *in vitro* studies with wear particles and leukocytes have focused on uncovering some of the intracellular signaling pathways involved in particle induced macrophage activation and inflammation.²⁴ Discovery of specific cascades involved in these processes could pave the way for specific pharmacological target sites to inhibit the adverse expression of bone resorbing and inflammatory cytokines.

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23 Animal Models of Orthopaedic Prosthetic Infection

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I. INTRODUCTION

In the United States, more than 200,000 primary hip and 200,000 primary knee arthroplasties are performed each year. Between 0.5% and 2.3% of them will become infected within 10 years (Table 1).¹ Sepsis following total joint replacement can have catastrophic results both physically and psychologically for the patients, leading to failure of the arthroplasty, possible amputation, prolonged hospitalization, and even death.⁸ Although the use of prophylactic antibiotics and greatly improved OR techniques have decreased the infection rate from an average of 5.9% in 1975 to 1.2% in 1993,¹ challenges still remain for better preventive and therapeutic measures.

TABLE 1
Infection Rates of Prosthetic Infection

First author, year	Total cases	Infected cases	% of infection*
Josefsson 1993 ²	835	13	1.6
Josefsson 1993 ²	853	9	1.1
Lidwell 1982 ³	5831	34	0.6
Lidwell 1982 ³	2221	52	2.3
Andrews 1981 ⁴	N/A	68	1.3
Nelson 1980 ⁵	580	6	1.0
Fitzgerald 1977 ⁶	3215	42	1.3
Eftekher 1976 ⁷	800	4	0.5

* numbers of infection cases/total cases $\times 100 = \%$ of infection.

Animal models of osteomyelitis⁹⁻¹¹ and foreign body infection¹²⁻¹⁹ have been well established by using the rabbit, dog, chick, guinea pig, and rat. The experience from these models, especially the models of foreign body infection, has been very helpful in designing an *in vivo* prosthetic infection model. Actually, a prosthetic infection model is an extension of the models of foreign body infection, with more attention to the effect of prosthetic materials, the use of bone tissue (instead of soft tissue), or the imitation of a human total joint replacement.

Several animal models have been reported for the study of orthopaedic prosthetic infection. They include total joint replacement, skeletal implant, and soft tissue model (Table 2). The soft tissue model is actually a foreign body infection model. Animal models have been mainly used for the studies of pathogenesis of bacteria, *in vivo* behavior of bacterial biofilm, effect of biomaterials on prosthetic infection rate, and the effect of infection on biomaterial surfaces.

II. HOW TO DESIGN AN ANIMAL MODEL OF PROSTHETIC INFECTION

A. ANIMAL SELECTION

A careful selection of an animal model for a study of prosthetic infection is the key for an ultimate result. Theoretically, to imitate a human situation it is better to use a large animal such as a sheep, goat, or dog, especially when attempting to design a joint replacement prosthesis as the implant. A dog femoral model has been used to test the influence of skeletal implants on incidence of infection and the preventive effect of prophylactic antibiotics.^{28,29} Animal models using goats or sheep have not been reported. The shortcomings of using large animals include the need for large housing space, difficulty of handling, and high costs.

Rabbit joint replacement models have been reported by using a specially designed femoral head prosthesis,²¹ interphalangeal joint prosthesis²² or the first metatarsophalangeal joint²⁰ for humans. A rabbit model using bone screws as implants was reported to evaluate the interaction between bacterial biofilm and antibiotics.²⁴ Implant site infection models by rabbit subcutaneous implantation were also reported.³⁰⁻³² More recently, rabbit implant models, such as femoral condyle cylindrical implant,³⁵ tibial intramedullary nailing,²⁷ and tibial plating²⁵ have been reported. Based on the literature and our own experience, rabbits are excellent for implant infection models because (1) the rabbit has a good joint size for even a total joint replacement, (2) it is more easily infected compared to dogs or rats, and (3) it is relatively economical. Small animals (rodents) are good for studying bacterial pathogenesis, implant site infection rate (which normally need large numbers of animals), or antibiotic effects. They have been widely used for foreign body infection models of different purposes.³⁹ Small animals which have been used include guinea pigs,¹⁹ hamsters,^{36,37} and mice.^{12-14,16,34}

TABLE 2
Animal Models of Implant or Prosthetic Infection

Models	1st Author (Year) ^{Ref.}	Animal	Model Description	Inoculation Routes	Bacteria	Number of Inoculated Bacteria*	Incubation Time
Joint replacement	Belmatoug 1996 ²⁰	Rabbit	Partial knee arthroplasty (tibial Intraarticular injection plateau) with silastic implant		<i>S. aureus</i>	10 ²⁻⁸ cfu/ml	—
	Southwood 1985 ²¹	Rabbit	Prosthetic femoral head replacement	Local inoculation or IV injection	<i>S. aureus</i>	10 ⁶⁻⁷ cfu local	—
Skeletal model	Blomgren 1981 ²²	Rabbit	Femur defect filled by cement or total knee replacement	Local inoculation or IV injection	<i>S. aureus</i>	10 ⁸⁻⁹ cfu IV 10 ⁸⁻⁹ cfu	—
	An 1997 ²³	Rabbit	Cylindrical implants inserted into lateral femoral condyle	<i>In vitro</i> colonization before implantation	<i>S. epidermidis</i>	In suspension of 10 ⁶ cfu/ml	1 hr.
	Isiklar 1996 ²⁴	Rabbit	Femoral intercondylar notch, drill hole, cancellous SS screw	Local inoculation	<i>S. epidermidis</i>	At least 10 ⁷ cfu per implant site	—
	Arens 1996 ²⁵	Rabbit	Tibial diaphyseal plating, then, local bacteria injection	Local bacteria injection	<i>S. aureus</i>	4×10 ³⁻⁶ cfu	—
	Sanzén 1995 ²⁶	Rabbit	Upper tibial implantation of cylindrical Ti and PMMA	Local injection into medull. canal through implants	<i>S. aureus</i>	9×10 ³⁻⁷ cfu/implant	—
	Melcher 1994 ²⁷ Petty 1985, 1988 ^{28, 29}	Rabbit Dog	Tibial intramedullary nailing Cylindrical implants inserted into proximal femoral canal	Local inoculation Local inoculation	<i>S. aureus</i> Three different bacteria	2×10 ³ –4×10 ⁷ cfu 10 ⁸ cfu	—
Soft tissue model	Nakamoto 1995 ³⁰	Hamster	Coated stainless steel wires placed subcutaneously	Incubation with bacteria before implantation	<i>S. epidermidis</i>	In suspension of 10 ⁷ cfu/ml	15 min.
	Chang 1994 ³¹	Hamster	Subcutaneous cylindrical SS or Ti implants on the back	<i>In vitro</i> colonization	<i>S. epidermidis</i>	10 ⁷ cfu in TSB [‡]	overnight
	Buret 1991 ³²	Rabbit	Silastic placed in subdermal tissue for biofilm study	Local inoculation <i>In vitro</i> colonization before implantation	<i>Pseudomonas aeruginosa</i>	10 ⁸ cfu/implant In suspension of 10 ⁷ cfu/ml	— 3 hours

* Number of bacteria injected or the concentration of bacteria in the incubation suspension.

† TSB = Trypticase soy broth.

Nevertheless, animal selection is a very important step for an *in vivo* prosthetic infection study. One should be very careful to choose an animal which will fit the purpose of the project.

B. IMPLANT FABRICATION

Based on the specific purpose, implants made of different kinds of materials and with different sizes and shapes have been used. Implants can be fabricated into or obtained as a cylinder,^{26,28,34,35} a metal wire,³⁰ a screw,²⁴ a tissue cage,³⁹ a bone plate,²⁵ an intramedullary nail,²⁷ a small joint prosthesis for human,^{20,22} or even a prosthetic joint component.²¹ Implants can be made with a smooth surface or porous coated. Most of the implants made for bone ingrowth study are cylindrical and could be used as implants for prosthetic infection.

C. BACTERIAL INOCULATION

Common pathogens isolated from human prosthetic infections should be used for most of the *in vivo* infection models, such as *S. aureus*, *S. epidermidis*, or less frequently *E. coli*, proteus, or some anaerobics. Bacteria can be delivered to the implant surface or its surroundings by (1) colonization of bacteria to the implant *in vitro* before implantation, (2) direct injection into the implant site, or (3) injection of the bacteria into the bloodstream.

The number of bacteria needed to produce an experimental infection varies from one bacterium to another. For example, less slime-producing *S. epidermidis* will be needed to produce an infection than non-slime producers. The route of inoculation makes a large difference too. The relationship between the dose of inoculum and the development of infection after prosthetic replacement has been studied in a rabbit model.²¹ Contamination of the implanted wound site with only a few bacteria (less than 50 *S. epidermidis*) likely will result in infection. It is difficult to induce infection when the operation was performed without insertion of a prosthesis (10^4 bacteria), which may suggest that the implant inhibits the defense system for dealing with the insult. It is difficult to produce an infection by inoculating the bacteria intravenously rather than locally and this will be more obvious if this inoculation is given three weeks after operation.^{21,22} Based on the numbers in Table 2, at least 10^3 cfu, or an average of 10^{5-8} cfu are needed to produce an implant site infection.

D. SURGICAL AND NECROPSY TECHNIQUE

Surgery should be performed under strict sterile conditions. The skin area of the incision should be shaved carefully and cleaned with 70% alcohol before transfer to the operating room. The skin area should be wiped with 7.5% Povidon-Iodine and 70% alcohol and then properly draped. Standard surgical technique should be employed throughout the operation. The wound should be washed with saline and closed securely in layers. Harvesting specimens also should be done under the exact same sterile conditions in order to obtain a valid microbiological evaluation.

E. AUTHORS' PREFERRED MODELS

1. Subcutaneous Model

The subcutaneous model is represented by the work by Chang and Merritt³¹ published in 1994. Syrian hamsters and small ($2-3 \times 10$ mm) metal cylinders were used. The implant was inserted into the scapular area through a small incision at the sacral region with the aid of a tube and trochar system. Bacteria were introduced into the implant sites by pre-incubation with the implant or by injection after the implant was placed. Seven days after the implant replacements, the animals were sacrificed and the implants and surrounding tissues excised and placed in test tubes containing normal saline and ground with glass beads to isolate bacteria from the implants and tissues. Then, the resulting suspension was plated on agar plates for bacterial colony counts.

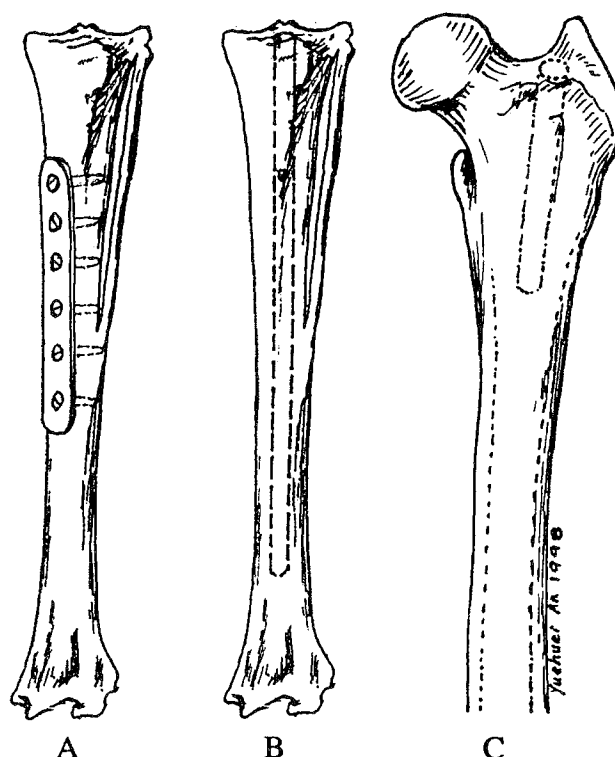


FIGURE 1. Illustrations of diaphyseal implant models for experimental prosthetic infection: (A) a tibia fixed with plate and screws in the rabbit;²⁵ (B) a tibia inserted with an intramedullary rod in the rabbit;²⁷ and (C) a canine femur inserted with an intramedullary rod.^{28,29}

2. Diaphyseal Bone Model

The rabbit tibial diaphyseal plating model by Arens et al.²⁵ and the rabbit tibial intramedullary nailing model by Melcher et al.²⁷ are appropriate for evaluating implant infections because these two fixation devices are the most commonly used implants in orthopaedic surgery. Another diaphyseal infection model is the canine femoral implantation of rods of different materials reported by Petty et al.^{28,29}

In the rabbit tibial plating model, the skin over the medial aspect of the midshaft of the tibia was cut under strict sterile conditions. Retraction of the muscles allowed bone exposure and then a bone plate (normally 6-hole plate) was fixed to the medial side of the tibia (Figure 1A). The wound was closed in layers and a plastic catheter was placed into the plate site through an operatively placed needle which was used for injection of bacterial suspension. The number of bacteria needed for a clinical infection ranged from 4×10^3 to 4×10^7 cfu/ml. Twenty-eight days after the surgery, bone samples were taken and ground into small pieces and cultured for bacteria.

In the rabbit medullary nailing model, through an anterior approach, the patellar ligament of the leg was divided and the medullary canal of the tibia opened using a drill (3.0–3.5 mm). The canal was then washed using a metal sucker and an appropriate amount of bacteria suspension was injected into the distal part of the canal. Then a 90 mm long and 3 mm diameter medullary nail was inserted into the tibia (Figure 1B). Twenty-eight days after the surgery, bone samples (distal half of the tibia) were taken and ground into small pieces and cultured for bacteria.

In the canine proximal femoral model,^{28,29} the femoral medullary canal was reamed through a drill hole at the greater trochanter. After introduction of bacteria using a Teflon tube, a 4 × 60 mm rod made of stainless steel, PMMA, or polyethylene was inserted into the proximal femur through the drill hole (Figure 1C). Animals were killed 15 days after surgery. Clinical, microbiologic and histologic methods were used to evaluate the occurrence of infection.

If a fracture is introduced in these models as in the clinical condition, the number of bacteria needed for an infection should be fewer than those cited in the reports.

3. Cancellous Bone Model

A cancellous bone model is represented by the animal models described by Isiklar et al.²⁴ and An et al.²³ They both placed the implants into the femoral condyle cancellous bone with bacteria injected into the implant site adhered to the implant (Figure 2). They are both intraarticular models with the implant inserted through intercondylar notch (screw)²⁴ or through the lateral condyle (the cylindrical implant).²³ The infection is showed by clear clinical septic arthritis and histologic peri-implant abscess. Another metaphyseal bone model is the rabbit upper tibial implantation of Ti cylinders reported by Sanzén and Linder.²⁶

In the screw model, through an anterior approach the distal femoral joint was exposed by dislocating the patellae laterally. A drill hole of 3.5 mm in diameter was created in the intercondylar notch. After bacterial inoculation in the drill hole, a stainless steel screw was placed into the femur. Four weeks later, quantitative bone cultures were performed to evaluate the infection.

In the lateral condyle plug model, a direct lateral approach to the left distal femoral condyle of each animal was made through a lateral incision and a drill hole equal in size to the implant (5 mm diam.) was made 5 mm proximal to the femoral-tibial joint articular surface and 5 mm posterior to the patellofemoral joint surface in a transverse direction. Care should be taken not to penetrate the medial cortex. The implants (with pre-adhered bacteria or without) were then inserted into the holes according to the experimental plan. The animals were sacrificed 28 days after the implantation and infection was diagnosed by clinical examination, histology, and/or a positive culture.

In the upper tibial plug model by Sanzén and Linder,²⁶ a PMMA or Ti cylindrical implant was implanted. After three weeks, $4 \times 10^{3-8}$ cfu *S. aureus* was injected into the medullary canal percutaneously through a central hole in the implant to create infection. After another four weeks, the animals were killed and radiographic, bacteriologic, and histologic methods were used to evaluate the infection.

4. Joint Replacement Model

The method described first by Blomgren²² in 1981 and then by Belmatoug et al.²⁰ is appropriate as a knee replacement model. They used joint prostheses for human finger joint replacement. The prosthesis used by Blomgren and the silastic nail-shaped implant by Belmatoug et al. have long stems which can be introduced into the diaphyseal part of the bone (Figure 3). Bacteria can be inoculated immediately after the implantation surgery or days or weeks later through local injection into the joint space or intravenously. The latter mimics the hematogenous prosthetic infection. The pathological and radiological characteristics of this model are close to those of human prosthetic joint infection. The model by Southwood et al.²¹ is also appropriate for studying joint prosthetic infection, but more effort has to be made for constructing the femoral component.

III. COMMONLY USED EVALUATION METHODS AND CRITERIA

A. CLINICAL FEATURES

It is difficult to get subjective findings from animals. Several abnormal behaviors may indicate a severe local or systemic infection, such as lethargy, less eating, a lack of weight gain, or weight loss. Physical signs are very important for judging an infection. Postoperatively, temperature should be taken and the wound observed daily for the first week and twice a week thereafter.

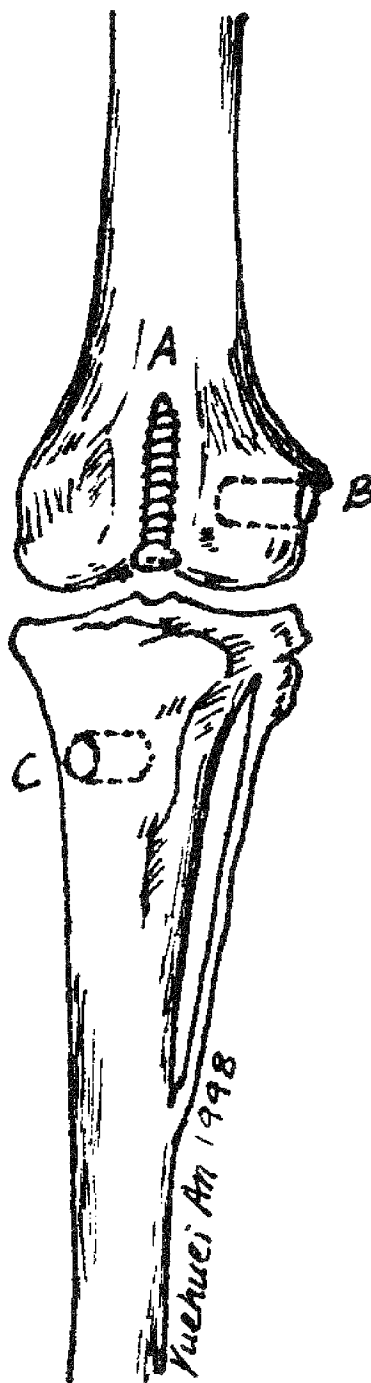


FIGURE 2. Illustration of rabbit cancellous bone implantation models for studying prosthetic infection: (A) an intercondylar implantation of a metal screw;²⁴ (B) a cylindrical inserted in the lateral femoral condyle of the rabbit;²³ and (C) a cylindrical implant inserted in the upper tibial metaphyseal area.²⁶

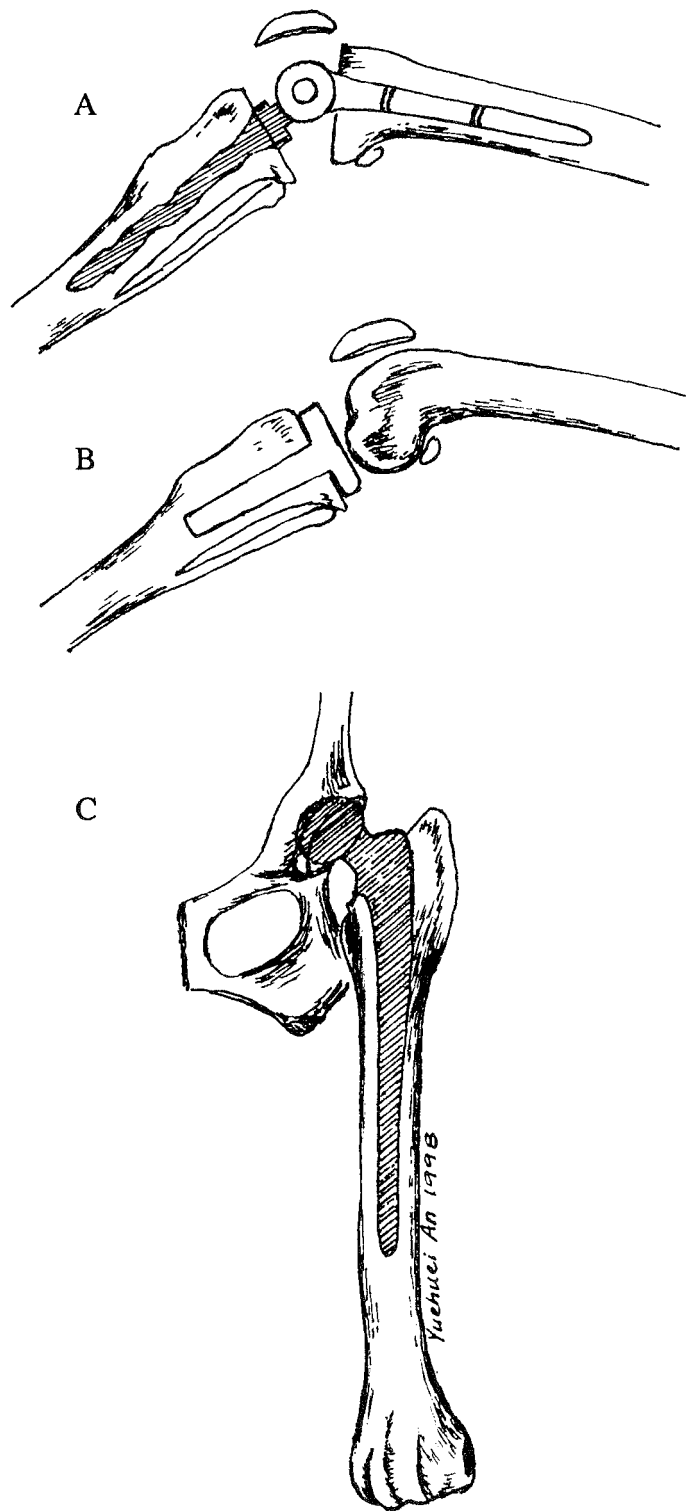


FIGURE 3. Illustrations of joint replacement models: (A) a total knee replacement with a St. Georg Finger-mittelgelenk Endoprothese;²² (B) a partial knee replacement with a Swanson great toe implant HP;²⁰ and (C) a potential partial hip replacement in the dog for studying prosthetic infection (A rabbit partial hip replacement model was reported by Southwood et al.²¹).

TABLE 3
Appearance of the Wound and Grading

Grade	Appearance	Score	Clinical meaning
A	No abnormal signs	0	No infection
B	Erythema and moderate soft swelling	0.5	Early infection or due to surgery
C	Large soft swelling or pus exudation	1.0	Definite infection
D	Pus exudation and systemic illness	1.5	Definite infection

TABLE 4
Radiographic Criteria

Variable	Definition	Score
Diaphyseal periosteal reaction	+/-present	1
	-/-absent	0
Osteolysis	+/-present	1
	-/-absent	0
Sequestrum formation	+/-present	1
	-/-absent	0
Joint effusion	+/-present, widening of joint space	1
	-/-absent	0
Soft tissue swelling	+/-present	1
	-/-absent	0

Temperature change is a sensitive parameter of a local or systemic infection. The appearance of the wound can be recorded and graded using a grading system modified from the one introduced by Petty et al.²⁸ (Table 3). Normally, infection can be confirmed by soft tissue swelling with pus exudate.

B. RADIOGRAPHY

For an intraosseous model, radiographs should be taken immediately after operation to check the implant position and at two-week intervals thereafter, until the animal is sacrificed. The development and progression of infection can be assessed using five criteria (Table 4), modified on the previous radiological descriptions of osteomyelitis in rabbits.⁹ They are (1) diaphyseal periosteal reaction; (2) osteolysis; (3) sequestrum formation; (4) joint effusion; and (5) soft tissue swelling. Using these criteria, a numerical score can be assigned and the six scores added together to give an overall ranking for radiographic severity. Radiography is a very useful method for diagnosing an infection in a diaphyseal area but is less favorable when a prosthetic infection is at an epiphyseal or metaphyseal location.

C. LABORATORY TESTS

For any abnormal wound exudate or sinus discharge, a swab should be taken for bacterial culture. It is realized that there are no direct relations between results of swab culture and clinical signs of infection. In a recent study by the authors using a rabbit femur implant infection model, there were only two positive swab cultures in 11 histologically diagnosed infections.²³ A culture in broth may yield more positive growth. Blood samples can be collected one day preoperatively and 3, 7, 14, and 21 days post-operatively for blood cell counts and erythrocyte sedimentation rates. Getting

TABLE 5
Histologic Criteria of Prosthetic Infection when Implant is Inserted into Cancellous Bone

Histologic findings	Diagnostic meaning
Inflammation with abscess formation	Infected
Presence of sequestrum (not drilling debris)	Infected, within abscess or near an abscess
Intracellular bacteria (Gram stain)	Infected, when found in an abscess, abscess capsule, or in inflamed tissues (often neutrophil or macrophage infiltrated area)
	Not infected, when found without evident inflammation or abscess
Inflammation with fibrosis	Infected, if bacteria found in inflamed tissues
	Not infected, if no bacteria found

information from blood sampling is sometimes not practical because some of the samples will be clotted (blood aspiration is often not fast enough for animals of this size) and the work is very time consuming.

D. BACTERIOLOGICAL STUDY

The explanted implants can be placed in PBS and agitated on a vortex mixer for five min. or sonicated for 30 min. to harvest bacteria for culture. Serial dilutions will be made and the solutions spread on a tryptic soy agar plate and added to tryptic soy broth for culture. Subcultures will be prepared at 24 hours if needed. The cultures will be considered positive for infection as described by Petty et al.²⁸ if (1) primary culture or subculture yields any bacteria that had been inoculated or (2) primary or subculture yield any bacteria. This criterion is subject to change according to the individual situation. If the implant is cylindrical, it can also be rolled over an agar surface and cultured overnight for bacterial colonies.

E. HISTOLOGICAL STUDY

The specimens can be embedded in paraffin (if the specimens are bone, they should be decalcified first) and multiple 4 mm thick sections cut. Implants should be removed before embedding. According to the authors' experience, this will not destroy the specimen. We found that abscesses were still intact after the implant was removed.²³ Adjacent soft tissues should be evaluated histologically because soft tissue abscess or drainage tract may exist. Draining lymph nodes also should be evaluated. Selected sections should be stained with Gram stain for detecting the existence of bacteria in the pus, the capsule of the pus, or in any inflamed tissues. The histological parameters in Table 5 can be used to evaluate the samples with implants inserted into cancellous bone. It is useful when an answer of "yes-or-no" is needed. The authors feel only the existence of microabscesses is definite evidence of histological infection (Figure 4).²³ Gram stain is useful for localization of bacteria in abscesses or inflammatory tissues (Figure 5). For the histologic criteria of experimental prosthetic infection involving a diaphyseal area, one should consult the work by Petty et al.²⁸

IV. APPLICATIONS OF PROSTHETIC INFECTION MODELS

A. PATHOGENESIS OF BACTERIA

When an animal model is employed, there are always questions, such as how many bacteria can cause prosthetic infection, how are they disseminated, can bacteria stay on the implant surface

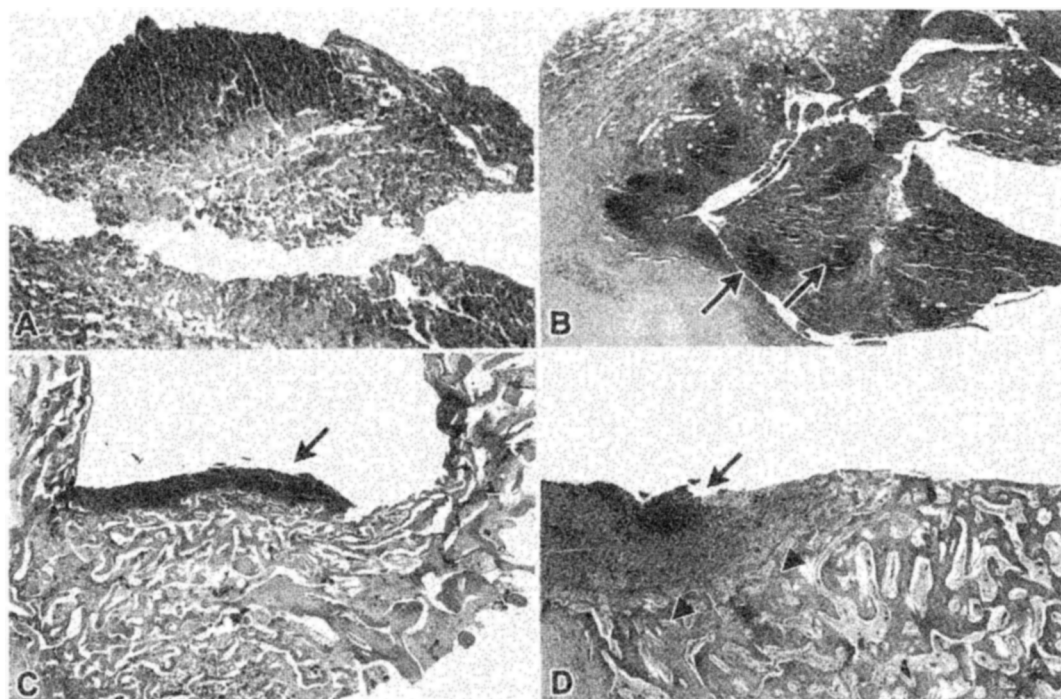


FIGURE 4. The histologic images show the abscesses found near the implant both in bone and soft tissue. (A) An abscess found in the soft tissue next to the lateral end of the implant. The imaginary location of the implant is under the lower part of the image. Part of the pus is separated from the lower part due to the tissue-processing procedure. (B) A sac of inflammatory exudate and pus found in the soft tissue covering the lateral end of the implant. The imaginary location of the implant is on the right side of the image. (C) An abscess found at cancellous bone next to the end of the implant. The implant was removed before sample embedding. (D) An abscess in the cancellous bone next to the side of the implant. The arrowheads indicate the borderline between the abscess and the surrounding bone. This curved borderline was created by erosion by the pus, so the bone surface was excavated away from the interface with the implant. (From An, Y. H., et al., *J. Bone Joint Surg.*, 79B, 816, 1997. With permission.)

for a long time without clinical infection,³³ and are the virulences of different bacteria the same? Zimmerli et al tested the effectiveness of different numbers of colony-forming units of *S. aureus* on the infection rate of a foreign body (tissue cage: a perforated tube).¹⁹ Blomgren verified the possibility of hematogenous bacterial dissemination of a total joint prosthesis and the subsequent infection.²² He also found that *S. aureus* (Wood 46) and *Propionibacterium acnes* (ATCC-6919) have the same ability to cause hematogenous infection of a total joint replacement. To find the effect of bacterial slime on the infection rate, Christensen et al reported a mouse foreign body infection model. Animals challenged with the slime-producing *S. epidermidis* developed three times as many infections as animals challenged with the strain that did not produce slime.¹² Also, animal models can be used to produce a bacterial biofilm for pathobiological study of implant surfaces.³²

B. *IN VIVO* BEHAVIOR OF BIOFILM

Buret and colleagues³² studied the morphology, ultrastructure, and microbiology of intact biofilm developing on an implant surface which was harvested from implants colonized with *P. aeruginosa* inserted into the peritoneal cavities of rabbits. Also in a rabbit model, Isiklar et al.²⁴ examined the penetration of antibiotics into biofilm formed by *S. epidermidis* following local and

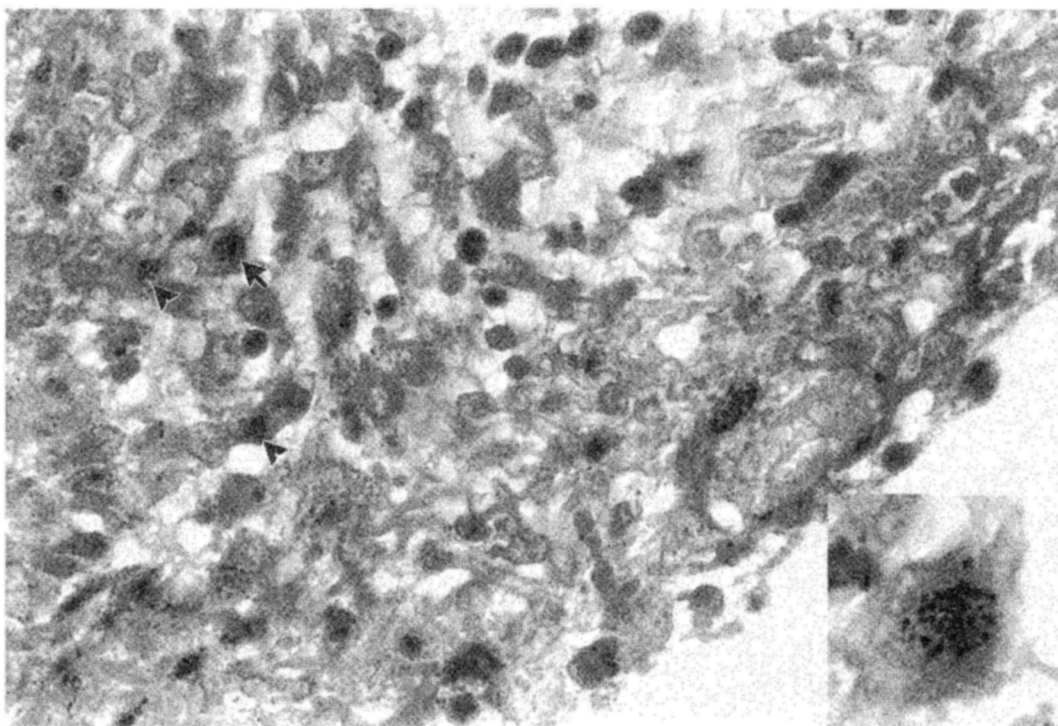


FIGURE 5. Gram stain positive bacteria (the arrow and arrow heads) were found in the cytoplasm of inflammatory cells within an abscess which was composed of polymorph leukocytes (neutrophils), macrophages, and degenerated or dead cells. The cell indicated by the arrow was enlarged to show details in the cell (inset). (From An, Y. H., et al., *J. Bone Joint Surg.*, 79B, 816, 1997. With permission.)

parenteral administration of vancomycin. This method is closer to the human situation because of the use of tibial bone, stainless steel implant, and the introduction of *S. epidermidis* which is a common pathogen for prosthetic infection.

C. EFFECT OF PROPHYLACTIC AND THERAPEUTIC ANTIBIOTICS

Animal models are excellent for studying the effects of prophylactic and therapeutic antibiotics on prosthetic infection because of the homogeneity of the animals for good comparison, reproducibility, and easily controlled time periods for observation.^{13,16,24,29} For example, Petty et al.²⁹ studied the preventive effectiveness of wound irrigation with normal saline, irrigation with saline containing neomycin, antibiotic-impregnated polymethylmethacrylate, and systemic antibiotics administration on prosthetic infection. The result showed that saline irrigation had no effect on infection rate; systemic use of cefazolin and neomycin irrigation slightly reduced the infection rate and the use of bone cement containing gentamicin caused a significant reduction.

D. EFFECT OF BIOMATERIALS ON PROSTHETIC INFECTION RATE

Several investigations on the effects of orthopaedic implants on the incidence of infection have been reported. Merritt et al.³⁴ designed a soft tissue model in the mouse and tested the implant site infection rates with porous and dense materials. They found that in the acute model the infection rate with the porous implant was greater, while in the chronic model after tissue invasion the infection rate with the dense materials was greater.

Petty et al.²⁸ established a dog model to evaluate the influence of skeletal implants on incidence of infection challenged by *S. aureus*, *S. epidermidis*, and *Escherichia coli*. The results showed that all of the implants (including stainless steel alloy, cobalt-chromium alloy, high density polyethylene, prepolymerized polymethylmethacrylate, and polymethylmethacrylate polymerized *in vivo*) were significantly more likely than the controls (medullary reaming only, without implantation and bacterial challenge) to be associated with infection, and polymethylmethacrylate polymerized *in vivo* was found to be significantly more likely than all other implants to be associated with *S. epidermidis* infection. In another study, the rabbit tibia was plated with steel or titanium plate. Under otherwise identical experimental conditions the rate of infection for steel plates (75%) was significantly higher than that for titanium plates (35%).²⁵

Using a rabbit model it was found that the physical configurations of intramedullary nails had significant effect on the implant infection rate. A much higher infection rate was in the group using slotted nails (59%) compared to the group of solid nails (27%).²⁷

The use of fibrinolytic agents to coat steel wires to decrease infection was reported recently.³⁰ In this study both the heparin coated and urokinase coated wires exhibited significantly decreased infection rates compared with uncoated wire. The mechanism of this effect may be the inhibition of bacterial adherence by heparin and fibrinolytic agents.

In the authors' laboratory, it has been demonstrated that albumin coating on titanium surface inhibited bacterial adhesion by 90%.³⁵ Then, coated (albumin) and uncoated titanium implants were exposed to a suspension of *S. epidermidis* prior to implantation into the lateral femoral condyle of the rabbit. According to the results, animals with albumin coated implants had a much lower infection rate (27%) than those with uncoated implants (62%). This finding may represent a new method for preventing prosthetic infection.²³

E. EFFECT OF INFECTION ON BIOMATERIAL SURFACES

It has been noted *in vitro* that implant-centered infection has certain effects on the behavior of prosthetic material, such as the corrosion of stainless steel surface,³⁶ or the destruction of hydroxyapatite coating.³⁷ Kieswetter and colleagues further studied the destructive effect of *S. aureus*, *S. epidermidis* or *Proteus* on the integrity of hydroxyapatite coating using an animal model, which consists of subcutaneous implantation of prosthetic materials in hamsters.³⁸ The results showed that significant destruction of HA coating can occur due to the growth of bacteria. Damage of HA coated implants appeared to be more severe *in vitro* than *in vivo*.

V. CONCLUDING REMARKS

Large amounts of research work have been done with great achievements in understanding the mechanisms of bacterial adhesion and prosthetic infection. Because of the potential tragic results and the large number of prosthetic procedures, prosthetic infection still remains a major challenge to biomedical researchers. For the complex nature of bacteria and their sophisticated interaction with biomaterials and host tissues, the various laboratory methods and animal models are very important and remain the major approach in solving this problem.

ACKNOWLEDGMENTS

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Part VI

Animal Models for the Study of Ligaments and Tendons



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24 Animal Models of Ligament Repair

Jason J. McDougall and Robert C. Bray

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I. INTRODUCTION

Once thought of as inert, vestigial structures, the ligaments of diarthroidal joints are now recognized as complex organs which play a vital role in articular physiology and in the maintenance of joint homeostasis. In addition to a crucial stabilizing function, ligaments have proprioceptive and nociceptive capacities as well as providing an efficient means of transmitting loads between bones with the aim of permitting dynamic and fluid movement. It becomes apparent, therefore, that if these tissues are injured and do not repair effectively, then normal locomotion and future joint structural integrity are compromised. Indeed research has shown that rupture of knee joint ligaments leads to instability with concomitant abnormal loading patterns.¹⁻³ Over time these abnormal loads could cause degeneration of joint tissues ultimately leading to the development of osteoarthritis.⁴⁻⁹

The study of ligament injury and subsequent repair in patients is problematic due to the complex nature of joint trauma. Activity-related injuries rarely involve damage to an isolated ligament but more often result in a number of ligaments being injured with an assortment of insults occurring

in other articular tissues. Hence, when investigating ligament repair, it is prudent to use animal models so that the type and degree of ligament injury can be controlled and the confounding influence of extraneous healing responses can be excluded. While vital for the investigation of ligament biology and function, caution must always be observed when interpreting the results of animal research since experimental responses may be species and ligament specific and, therefore, may not always be representative of the human condition. This limitation aside, it is hoped that by repeating experiments in a variety of different species basic trends in joint physiology and pathophysiology will eventually be uncovered. This approach to animal research would render it more persuasive when extrapolating to human conditions.

II. SPECIES COMMONLY USED IN LIGAMENT RESEARCH

The choice of species used in research is usually dictated by non-scientific factors such as cost and the ability of the institution to house certain types of animals. While these are often valid restrictions when choosing an animal model, ideally the investigator should attempt to select the species which is most relevant to the questions posed. Limiting a study to only “tried and tested” models of healing is often unproductive since a result found in one species does not necessarily translate to the same result occurring in all species. A number of different animals have been used in the study of ligament repair of which the most frequently used are outlined here along with their relative advantages and disadvantages.

A. DOGS

Some of the earliest work on ligament healing was performed on dogs¹⁰⁻¹² and this species remains one of the most popular animal models to date.¹³ The reason for their prevalence in this area of research is mainly due to the fact that dogs are easy to handle and are generally receptive to various exercise regimens. In addition, since an extensive database has been established from work performed in the veterinary field, researchers have a wealth of published information on which to call. The downside of using dogs in ligament research is the obvious emotive issues involved and the consequent restrictions placed on the investigator by the host institution.

B. RABBITS

Rabbits are widely used in biomedical research due to their docile nature and relatively inexpensive purchasing cost and upkeep. The biochemical and functional properties of rabbit knee ligaments have been well documented (For reviews see references 14-16) and, therefore, this model provides a strong scientific base from which to study ligament healing. The rabbit lends itself to medial collateral ligament (MCL) repair studies since this ligament is well developed and easily accessible in this species. In contrast, rabbit intraarticular ligaments are small and fairly inaccessible making the study of cruciate ligament healing difficult in this particular animal. Several laboratory strains are available; however, it should be noted that some such as the NZW rabbits are prone to obesity during long-term caging and the loads on their joints may be abnormally high.

C. GOATS AND SHEEP

The larger joint sizes in these animals mean that they are ideally suited for cruciate ligament healing studies. Many investigators are now using sheep and goats to assess cruciate ligament reconstructions¹⁷⁻²¹ and ligament prostheses.²²⁻²⁵ Another possible advantage of using these large animals is that the degree of flexion in their stifle joints is less than in rabbits and dogs suggesting a closer analogy to human knee joints. On the downside, the practical and economical considerations are evident with the animals requiring special housing facilities and elaborate post-operative care.

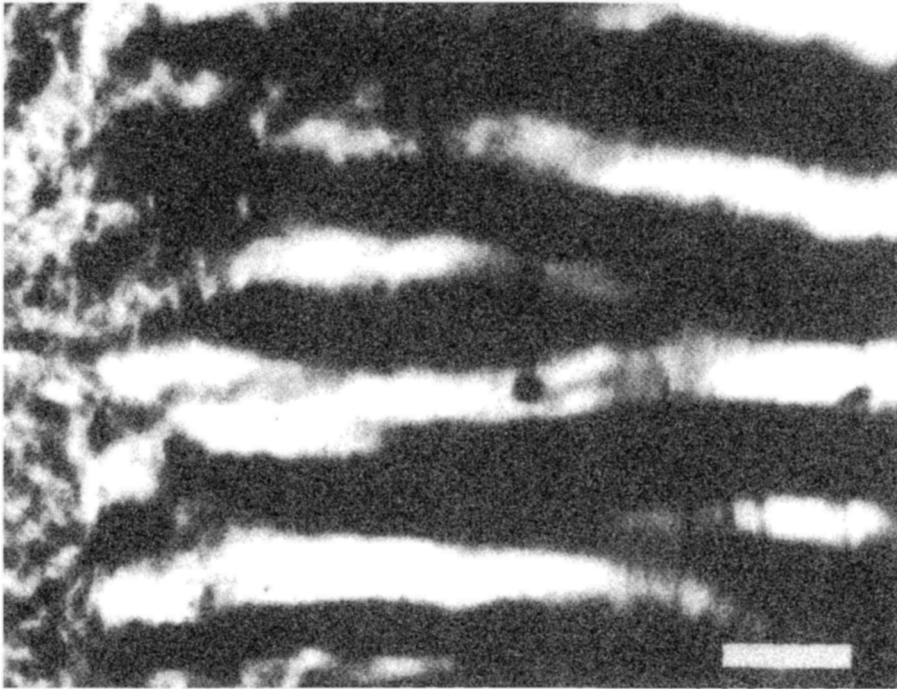


FIGURE 1. Photomicrograph of rat medial collateral ligament taken using polarized light. Longitudinally, the collagen fibres appear to have a sinusoidal repeating pattern often referred to as “crimp.” Scale bar = 50 μ m.

D. RATS

Despite their low cost and ready availability, rats are not generally used in ligament research. In addition to the small size of their joints and the practical encumbrances size imparts, the rat musculoskeletal system continually grows throughout its lifetime such that the bone and articular soft tissues are in a constant state of remodeling. Hence, interpretation of functional and biological outcomes in this species is unreliable.

III. AN OVERVIEW OF NORMAL LIGAMENT BIOLOGY

In order to appreciate ligament healing models more fully, a basic understanding of normal ligament structure and function must first be realized. As defined by *Gray's Anatomy*,²⁶ ligaments are bundles of white fibrous tissue serving to connect together the articular extremities of bones. Structurally, these tissues are made-up of bundles of collagen fibers which are arranged parallel to the longitudinal axis of the ligament and are mounted in a coarse tissue matrix. Polarized light microscopy reveals a regular sinusoidal pattern along the length of these collagen bundles, a phenomenon which has been described as “crimping” (Figure 1). The significance of this concertina-like pattern is unclear but it is thought to act as a mechanism to accommodate forces, allowing loads to be dissipated before structural damage occurs.^{14,27}

Biochemical analyses show that the major component of connective tissues is water accounting for some 70% of the wet weight of the tissue (Table 1). The function of water in ligaments is somewhat moot; however, it has been suggested to be necessary for the intracellular transportation of ions as well contributing to the viscoelastic behavior of the tissue.^{28–30} The next most abundant component is collagen, of which type I collagen is the most ubiquitous. Collagen provides the structural mainstay of ligaments by providing a source of stability and mechanical integrity to the tissue. The remaining constituents (elastin, fibronectin and proteoglycans among others) are all

TABLE 1
The Relative Biochemical Constituents of Normal and
Scarred Rabbit Medial Collateral Ligament Tissue

Component	Normal ligament proportion (%)	Scar ligament proportion (%)
Water	70	75
Type I collagen	20	12
Other collagen types	3–5	5
Elastin, fibronectin, and glycoproteins	2–4	6
Proteoglycans	<1	1–2

matrix elements whose functions continue to be defined. Proteoglycans are thought to act as “shock absorbers” by altering ligament viscoelasticity via their water binding properties, while elastin may be responsible for the elastic properties of ligaments.^{14,15} Further research is required to elucidate the exact role of these substances in normal ligament function so that their levels and chemical composition can be optimized during post-traumatic repair.

IV. SPECIFIC MODELS OF LIGAMENT HEALING

When studying ligament repair *in vivo*, the tissue in question must first be subjected to a certain degree of trauma before it can heal. Therefore, the models used in this area of research also tend to be used in other investigations of soft tissue injury. The limitation in variety and scope of the major models available reflects the rudimentary nature of this type of research and the need for more innovative concepts so that the intricacies of ligament healing can be probed more deeply.

A. LIGAMENT DIVISION

When ligaments fail, they do so by varying degrees of tearing of their collagen bundles. In microfailure the ligament undergoes only partial tearing, often referred to in layman’s terms as a sprain, while greater loads result in catastrophic failure in which the entire ligament is severed. Hence, models in which the ligament is divided are often a good approximation to clinical injuries and as such have formed the basis of ligament healing studies. Some of the models of ligament division and repair are summarized in Figure 2.

1. Mechanical Rupture

The seminal work on ligament injuries attempted to replicate the clinical manifestation of trauma by manually subjecting the joint to forces which were in excess of normal physiological limits. Some of the earliest reports of injury induction involved violent abduction of the knee.^{31–33} This rather crude injury model resulted in ligament tears which tended to occur near the entheses although the extent and specific location of the rupture varied.³⁴

Later studies attempted to refine ligament ruptures by controlling for the site and level of tissue damage with minimal inclusion of other articular structures. Frank et al.³⁵ produced a “garrotting” model in which a 3-0 braided steel suture was passed under the MCL and then pulled in a firm, upward movement perpendicular to the joint. The resultant level of tissue damage was consistent between animals and was found to be restricted to the site of rupture in the ligament midsubstance. Variations of this technique have included passing a pair of hemostats³⁶ or a 2.5-mm diameter

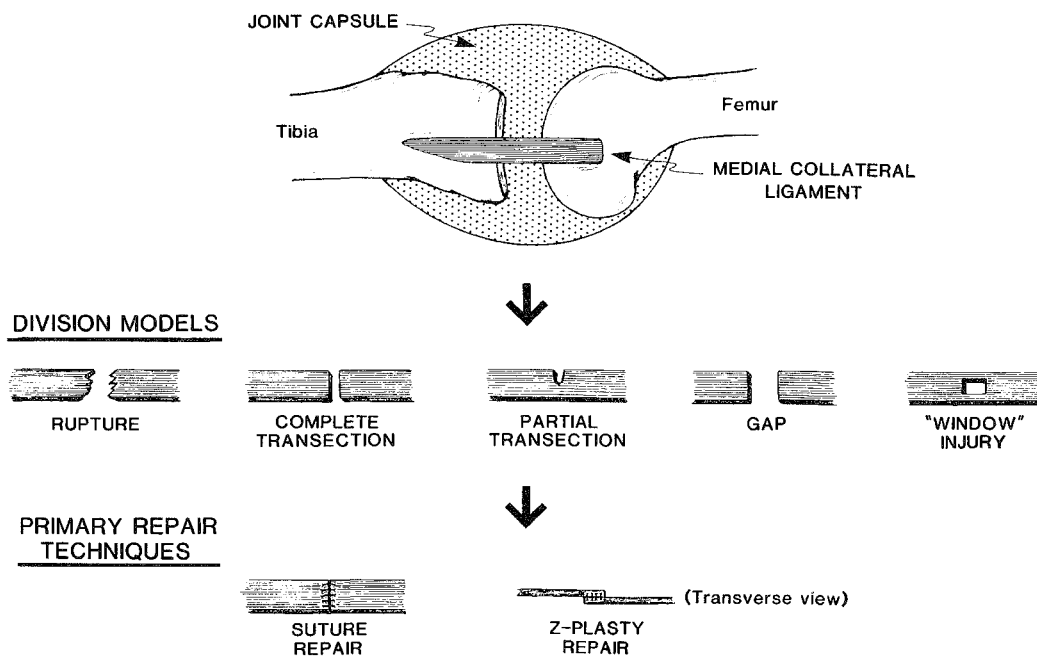


FIGURE 2. Common models of division and repair.

stainless steel rod³⁷ under the ligament before sharply pulling the implement medially with such force so as to cause a midsubstance rupture.

The morphology of this injury model is similar to the clinical presentation of failure in that the torn ligament ends are frayed and irregular. Although clinically relevant, these injury models still incorporate a significant number of uncontrolled variables such as mechanical disturbance of the ligament in areas remote from the injury site. This inherent complexity of all rupture models means that their use in laboratory investigations should be viewed with a degree of skepticism because of the difficulty in interpreting their effects.

2. Surgical Transection

To obviate some of the concerns associated with rupture models, a less invasive method of injury has emerged, viz., surgical transection of the ligament with a scalpel. Complete transection involves a simple transverse midsubstance division of the ligament resulting in a uniform, regular cut.^{10,11} It was originally thought that effective ligament healing could only occur when, as in the rupture model, the separated ends were frayed providing a greater surface area for healing to take place. This belief cast doubt on surgical transection as a viable model of ligament repair. However, Chimich et al.³⁸ showed that the histological and mechanical properties of a healing MCL were not affected by the configuration of the torn ligament ends, i.e., it didn't matter whether the ends were sheer as in surgical transection, or whether they had the "mopped" appearance of having been ruptured.

It has been postulated that apposition of torn ends may be a prerequisite for the functional recovery of healing ligaments by minimizing the development of potentially inferior scar tissue and maximizing proliferation of dense collagenous material within the healing zone.^{12,39,40} To examine this proposition, two distinctive injury-repair models were created in the rabbit MCL.⁴¹ The first was a Z-plasty apposition model in which the ligament was transected in a sagittal plane

and the ends overlapped before suturing them together. This procedure maximized the contact area between the two sections of ligament thereby minimizing the amount of scarring. The second model aimed to favor scar production by maintaining separation between the MCL ends. This was achieved by excising a 4-mm segment of tissue from the ligament midsubstance leaving a discrete gap. After 40 weeks of healing, both injury types had remodeled culminating in the formation of a scar, the level of which was, as predicted, greater in the gap model than in the contact state. In addition, gap scars could be distinguished from old ligament whereas Z-plasty scars presented as a mixture of original ligament and neoligamentous scar tissue. Thus gap injury provides a unique model for the study of pure ligament scar without the confounding influence of old tissues infiltrating from surrounding regions.

Numerous other techniques have been developed to promote apposition of ligament ends especially in the anterior cruciate ligament (ACL) where the ligamentous stumps rarely unite through scar tissue and, consequently, atrophy. One of the simplest methods is by primary repair where the ligament ends are brought together by suturing.⁴² Although several different approaches have been examined, the ligament ends refuse to bond and healing is eluded. As found in tendon repair studies,^{43,44} possible reasons for this lack of ACL healing may be that the type of suture material and stitch pattern used were unsuitable for this tissue. Alternatively, it may be that the forces applied to the ligament during recovery were in excess of what the sutures could handle and ligament stump retraction was inevitable. Therefore, a specialized gap model was created whereby only a mid portion of tissue was removed to produce a window-like injury.⁴² This procedure had the advantage of creating a restricted gap injury while retaining mechanical stability. Even though all of these countermeasures were considered, only 5% of these ACLs showed a positive healing response.

Partial ruptures account for nearly a third of all human ACL injuries leading researchers to develop a hemitranssection model of injury in which only a limited number of collagen bundles are cut. Evidence exists to suggest that ACL healing with improved functionality can occur in these models although the recovery period is somewhat protracted compared to other ligaments.^{12,45,46} In contrast, other studies have been unable to find any sort of healing response in this model even after trying to consolidate the laceration through primary repair.^{42,47} The outcome of these studies only reaffirms that a viable and reproducible model of ACL healing still evades the research community.

3. Microtrauma by Proxy

Following ligament rupture, the affected joint becomes lax and unstable leading to an alteration in joint mechanics.¹⁻³ The forces which are then placed on the joint have to be redistributed throughout the remaining uninjured ligaments leading to the generation of abnormally high loads in these structures. In the absence of any sort of intervention or attempts at repair, the uninjured ligaments may themselves undergo low level failure thereby exacerbating joint instability.^{48,49} One of the commonly used models to study secondary microtrauma is to assess the properties of the MCL in an ACL deficient knee. Normally associated with osteoarthritis research, transection of the ACL also provides a unique means of examining MCL integrity in a mechanically unstable environment. One of the first attempts to divide the ACL was performed by Pond and Nuki⁵ and involved a blind stab incision to the anterior surface of the joint. A scalpel blade was forced between the femoral and tibial condyles into the joint space in the hope of completely severing the ACL. One of the main drawbacks to this technique is the uncertainty of whether the entire ligament has been transected, not to mention the additional trauma exacted on the patellar ligament and infrapatellar fat pad. A less invasive and more reliable approach to ACL transection is by isolating the ligament following an arthrotomy. This technique entails making a longitudinal incision along the subpatellar region of the joint, reflecting the infrapatellar fat pad, isolating the ACL and then

surgically transecting it with a scalpel blade before finally closing the wound with sutures. In this way the investigator may be confident that the whole ligament has been severed with minimal disruption to other articular tissues.

B. LIGAMENT RECONSTRUCTION AND REPLACEMENT

In light of the ineffectiveness of apposition to promote functional recovery of injured ACLs, alternative treatment regimens have been sought. The philosophy that “if it doesn’t heal replace it” has gained popularity recently, so much so that reconstruction or replacement of the torn ACL with various biological or prosthetic materials has become common practice. Autografting uses tissues such as patellar ligament, semitendinosus tendon, quadriceps tendon or even meniscus to reconstruct torn cruciates. Since the graft material is harvested from the recipient animal, then there is a certain level of morbidity associated with the donor site. Allografts obviate this problem by removing these tissues from an independent donor animal; however, the disadvantages of this approach include the risk of infection and possible rejection of the graft. Preservation of the substitute ligament following removal from the host has also plagued the clinical use of allografts. Animal studies have shown, however, that freeze-drying the graft for up to a year does not appear to have a detrimental effect on the mechanical viability of the transplanted tissue.^{50–54}

Synthetic ligaments have been used to investigate the effect of supplementary support during cruciate ligament repair. One type of artificial ligament is the augmentation device which was designed to allow joint loads to be shared during autologous ligament healing or graft establishment.^{55,56} The protective effect the prosthesis imparts is only supposed to be temporary, with it carrying the greatest amount of load immediately after implantation and then gradually transferring load onto the repaired tissue so that it may eventually become a more functionally competent structure.

C. OTHER MODELS

Injured joints are conventionally immobilized to protect the repaired or reconstructed ligament from potentially damaging forces. Immobilization of experimental animals is usually carried out by fitting either a cast, brace or splint to the recovering limb. Although not a model of ligament repair *per se*, joint restraint in conjunction with one of the injury/repair models enables researchers to test the relative benefits of immobilization and remobilization on the postoperative care of healing ligaments.

To our knowledge, *in vitro* models of ligament repair are scarce, reflecting a glaring deficiency in this area of research. One of the few models available was developed by Witkowski et al.⁵⁷ to study the migration of ligament cells into a manufactured wound. The technique involves culturing human MCL or ACL fibroblast cells *in vitro* and then streaking them with an inoculating loop thus creating an acellular region. Different mediators can then be assessed for their healing properties under an array of conditions by adding them to the culture medium and monitoring their effect on the migration of cells into the wound.

V. EVALUATION OF HEALING RESPONSES

In order to appraise the effectiveness of the various repair techniques and the influence of the circumstances in which they occur, a multitude of different protocols are employed which span a myriad of disciplines. By performing the evaluations in normal as well as healing ligaments, the level of tissue recovery can be ascertained. This section describes some of the assessment procedures encountered and highlights some of the differences found between a ligament which shows a good healing response (MCL) and one that does not heal effectively (ACL).

A. HISTOLOGY

Ligament structure may be characterized by examining sections of the tissue histologically with haematoxylin and eosin stain. The appearance of ligament cells and collagen organization/size provide a strong indication as to the status of the tissue with respect to metabolic and mechanical parameters. As mentioned earlier, when viewed under polarized light, normal ligaments possess a “crimped” appearance resulting from the coherent organization of collagen fibers. In the scar region of a gap injured MCL, this repeating pattern is lost and the collagen fibers appear disorganized (Figure 3).

In transected ACLs, histological techniques failed to find any sign of healing even following primary repair of the lacerated ligament ends.⁴² With the advent of ACL reconstruction, attention has turned to the morphological analysis of healing grafts. In experiments using the patellar ligament autograft, it was found that there was an initial phase of avascular necrosis followed by radical remodeling of the tissue such that the graft eventually had the histological appearance of a normal ACL.^{58–60}

Immunohistochemistry has been used to detect neuropeptidergic nerve fibers in normal and healing MCLs.^{61,62} Neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) are pro-inflammatory agents released from the terminals of afferent nerves and which cause vasodilatation and protein extravasation. In addition to their haemodynamic effects, neuropeptides possess trophic properties causing neovascularization and tissue remodeling by promoting fibroblast differentiation. Experiments have shown⁶² that six weeks after gap injury, SP and CGRP immunoreactivity is increased in the healing scar tissue although the nerves appear truncated and tangled (Figure 3). Increased presence of peptidergic nerves in the MCL may relate to the sound healing potential of this structure.

B. BIOCHEMISTRY

Organ and cell culture has been used to investigate the biochemical properties of normal and remodeled ligament tissue. The process involves creating an injury to the ligament *in vivo* and then incubating the tissue *in vitro* so that biochemical analyses can be carried out. Examinations usually center on the remodeling processes associated with MCL scar formation, i.e., matrix component synthesis and the formation of various proteases responsible for the removal or structural alteration of these matrix constituents. In ligaments, type I collagen is believed to have a significant role in the biological and biomechanical properties of the tissue¹⁴ and as such a significant amount of biochemical research has been devoted to the mechanisms which control its production. It is thought that the functional outcome of healing ligaments may be improved by promoting the synthesis of the appropriate collagen type and optimizing its organization within the matrix. The specific biochemical parameters which are assessed, therefore, include collagen typing, crosslink analysis, fibril diameter and ground substance content.

Biochemical analysis of MCL scar tissue shows it to have an inferior composition compared to normal ligament, especially with respect to lower type I collagen levels (Table 1). The increased water and elastin content of ligament scars is thought to contribute to the decreased stiffness of the tissue (see below) while collagen fibril diameter does not appear to be applicable since size is unaltered in healing connective tissue.⁶³

The effect of injury on ACL biochemistry is still poorly understood; however, some information relating to this subject has started to emerge. Collagenase activity has been found to increase by 82% in transected rabbit ACLs compared to controls⁶⁴ providing a possible explanation for the resorption of torn ACL ends. The fact that ACL healing is limited even after apposition of the ligament stumps suggests that there is something intrinsically different about the make-up of this tissue which inhibits remodeling and scar production.

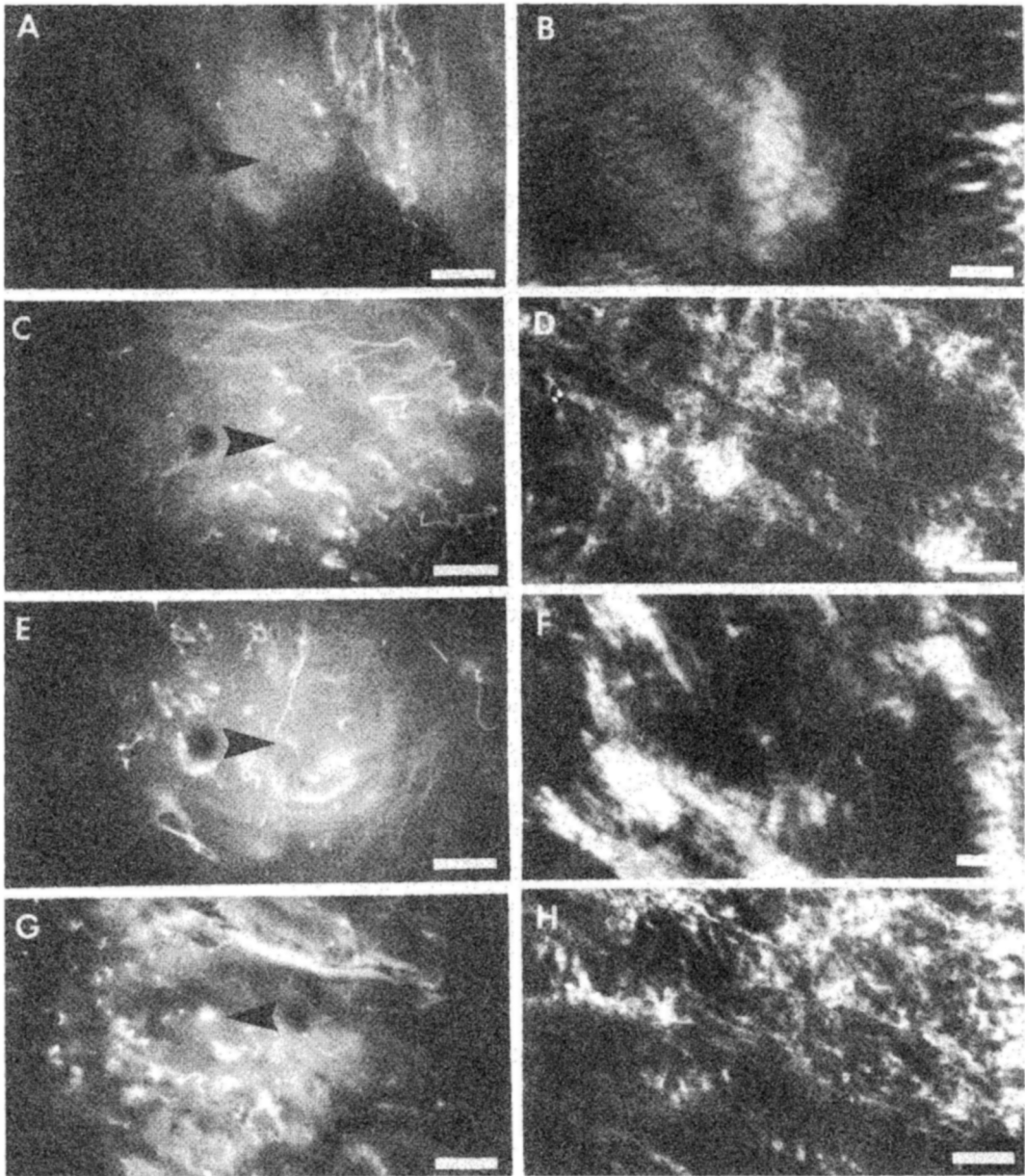


FIGURE 3. Fluorescence micrographs of CGRP- (A,C,E) and SP- (G) like immunoreactivity in the adult rabbit medial collateral ligament six weeks after gap injury. Micrographs B, D, F, and H are corresponding regions taken using polarized light. Note that the “crimping” effect has been replaced by disorganized, amorphous scar tissue. In the immunofluorescent images, the black dots represent suture markers placed to delineate the extremities of the gap injury and the arrows point to the healing zone. The peptidergic nerves are abundant and appear as a tangled mesh of truncated, varicose fibres in the scar tissue. Scale bar = 50 μ m. (From McDougall et al., *Anat. Rec.*, 248, 29, 1997. With permission.)

C. BIOMECHANICS

Functional assessment is the most widely used measure of post-traumatic recovery in connective tissue research. Innumerable protocols have been developed to test everything from the material properties of ligaments to the mechanical characteristics of bone-ligament-bone complexes. To

date, the majority of ligament biomechanics is performed *ex vivo* and, therefore, this evaluation method has little physiological significance. To account for this limitation, researchers have spent many years devising standardized protocols which attempt to control for biological factors such as temperature, specimen orientation and selection of suspension medium. This section attempts to outline some of the more pertinent findings but for an extensive coverage of this subject the reader is directed elsewhere.^{16,65,66}

The straightforward approach of merely mounting a section of ligament in a materials testing device is problematic due to the difficulty of securing the ligament ends without altering the properties of the tissue. Furthermore, one is never quite sure whether the clamp-tissue interface is being tested rather than the tissue itself. A more appropriate action is to leave the ligament attached to the bones which can then be clamped into the apparatus. In addition to improved clamping of the tissue, this bone-ligament-bone complex provides a structural unit for testing under tensile loading conditions. Once mounted, load is applied to the ligament and the effect on mechanical behaviors such as ligament stress and strain can be determined. Mathematical analyses of load-deformation plots provides a host of information relating to the functional properties of the ligament. The manner in which the load is applied to the structure (e.g., loading rate, loading level, or whether it is cyclic or not) is also of utmost importance when interpreting mechanical behavior. With regard to developing a sense of tissue biomechanics, cyclic low-load testing may provide the most physiological representation of ligament function under normal conditions.

Load-deformation curves carried out on healing MCLs have shown that the structural properties of bone-healing ligament-bone complexes are initially inferior to control but show progressive improvement at 6 and 12 weeks post-injury.^{15,16,66} Interestingly, the material properties of a healing MCL, of which tissue stiffness is an example, were consistently reduced compared to normal even at 14 weeks. This means that under these conditions the bone-healing ligament-bone complex undergoes a certain degree of functional recovery despite a decline in ligament substance integrity. The reason for this apparent recovery paradox is unclear but may be related to compensatory changes occurring at the entheses.

The effect of treatment on injured MCLs has also been studied extensively. Z-plasty repair of torn ligament ends resulted in a significant increase in ligament strength compared to untreated gap injuries⁴¹ reaffirming the benefit of apposition in ligament healing. Immobilization of an injured joint also has significant repercussions on the composition and material properties of neoligamentous scar tissue. Experiments performed on severely injured rabbit knees, i.e., in which there is a composite MCL gap/ACL transection injury, showed that the greater loads associated with joint mobility produce an increase in MCL cross-sectional area; however, load-deformation characteristics of the ligament were the same as in controls.⁶⁷ These findings are similar to other reports in which only the MCL was transected.^{68,69} The structural properties of healing MCLs which had been subjected to a primary repair with immobilization regimen were found to be poorer than those of both control and untreated ligaments. Taken together these data show that immobilization of injured ligaments is obstructive to the long-term recovery of normal MCL function and that suture repair may only be effective when some modicum of joint stability is maintained via an intact ACL.

Three years after partial transection of the goat ACL, the stiffness and tensile strength of the structure were not significantly different from control values suggesting that functional recovery may be possible in this tissue after all.⁴⁶ The reason given for this apparent healing response was that since only the posterolateral bundle was severed, the anteromedial fragment acted as an internal splint providing non-surgical apposition of the ligament stumps. This finding is interesting since numerous attempts at ACL apposition in the complete transection model failed to generate repair responses in this tissue. It is possible that the introduction of ligatures at the site of injury could restrict blood flow to the area thereby preventing the delivery of necessary healing mediators.

When mechanically testing reconstructed ACLs, factors such as graft position, fixation protocol, initial graft tension and postoperative rehabilitative regimens must all be considered. Ultimate load and linear stiffness of newly implanted patellar ligament autografts in dogs have been shown to be

about 10% of control bone-ACL-bone complexes.^{56,70} Although high-load structural properties of autografts continue to improve with time, complete recovery is never attained even after three years.⁷¹ The use of allograft tissue for ACL reconstruction also exhibits poor functional recovery in other animal knees. As alluded to earlier, cryopreservation of nonautogenous tissue does not seem to affect the material properties of the graft; however, there is some evidence to suggest that some of the techniques used to sterilize the implant may be detrimental.⁷²

As an aside, it should be noted that the contralateral limb served as the control in all of the preceding studies and, hence, a possible bilateral effect of joint injury could be prejudicing these results. Reinvestigation using normal animals as controls may be required before any of these results can be fully accepted.

D. VASCULAR PHYSIOLOGY

Of all of the classic signs of soft tissue healing, none are more conspicuous than those of the microvascular system. Hyperemia, angiogenesis and increased vascular permeability are all synonymous with tissue healing which makes it all the more remarkable that this area of research has been overlooked for so long. The emergence of innovative and readily applicable techniques will only serve to enhance our understanding of joint vascular physiology providing information on one of the most exciting and scientifically pertinent areas of ligament research.

Several methods of measuring ligament blood flow have been developed including hydrogen clearance,⁷³ microsphere distribution^{74,75} and laser Doppler perfusion imaging.^{76,77} Bray et al.⁷⁵ found that MCL gap injury caused a significant rise in ligament blood flow at three and six weeks post-trauma. This hyperemia may lead to an increase in ligament hydrostatic pressure which, in conjunction with inflammatory extravasation, could explain the high water content of MCL scars and, hence, the decreased stiffness of remodeled ligaments. It follows, therefore, that if the vascular responses to ligament injury could somehow be controlled then the water content of the healing ligament could be moderated, possibly leading to a functionally more normal tissue.

McDougall et al.⁷⁷ found that the rabbit knee joint is unable to autoregulate and is wholly dependent upon extrinsic mediators to control its blood supply. Factors known to alter rabbit MCL blood flow include CGRP, adrenaline and the perivascular effects of the sympathetic nervous system.^{76,77} When the joint is made unstable through ACL transection, these vasoregulatory mechanisms fail and the MCL is deprived of a balanced blood supply.^{78,79} This loss of joint homeostasis may mean that the metabolic needs of the MCL are not being met which could account for the deterioration of the ligament in this model.

To date, measurement of graft revascularization and scar angiogenesis has relied upon qualitatively assessing histological sections of the relevant tissues. This approach is somewhat inadequate since no information is afforded regarding the number of new vessels or whether indeed they are patent with the existing capillary network. In our laboratory we have developed a novel technique which quantifies the volume of vessels supplying various articular structures.⁸⁰ By comparing the vascular volumes in a group of normal animals with those found following some sort of intervention, angiogenic activity would be represented as a measurable increase in tissue vascularity. The technique is based on spectrophotometrical analysis of intravascularly injected carmine red dye to give a measure of tissue vascular volume. We have used this methodology to investigate changes in rabbit knee joint vascularity following ACL transection. As shown in Figure 4, compared to sham operated controls, angiogenesis only appears to occur in the ipsilateral MCL of the unstable joint. Thus, microtrauma by proxy to the ipsilateral MCL stimulates vascular remodeling of the tissue which if extensive enough, may cause disorganization of collagen fibrils or even replace a portion of them altogether. This disruption to the collagen crimp pattern could weaken the MCL exacerbating joint instability. Interestingly, the lack of new vessels being formed in the infrapatellar fat pad or synovium may herald a possible explanation for the inadequacies of ACL healing since the bulk of the vasculature which nourishes the ACL arises from these tissues.⁸¹

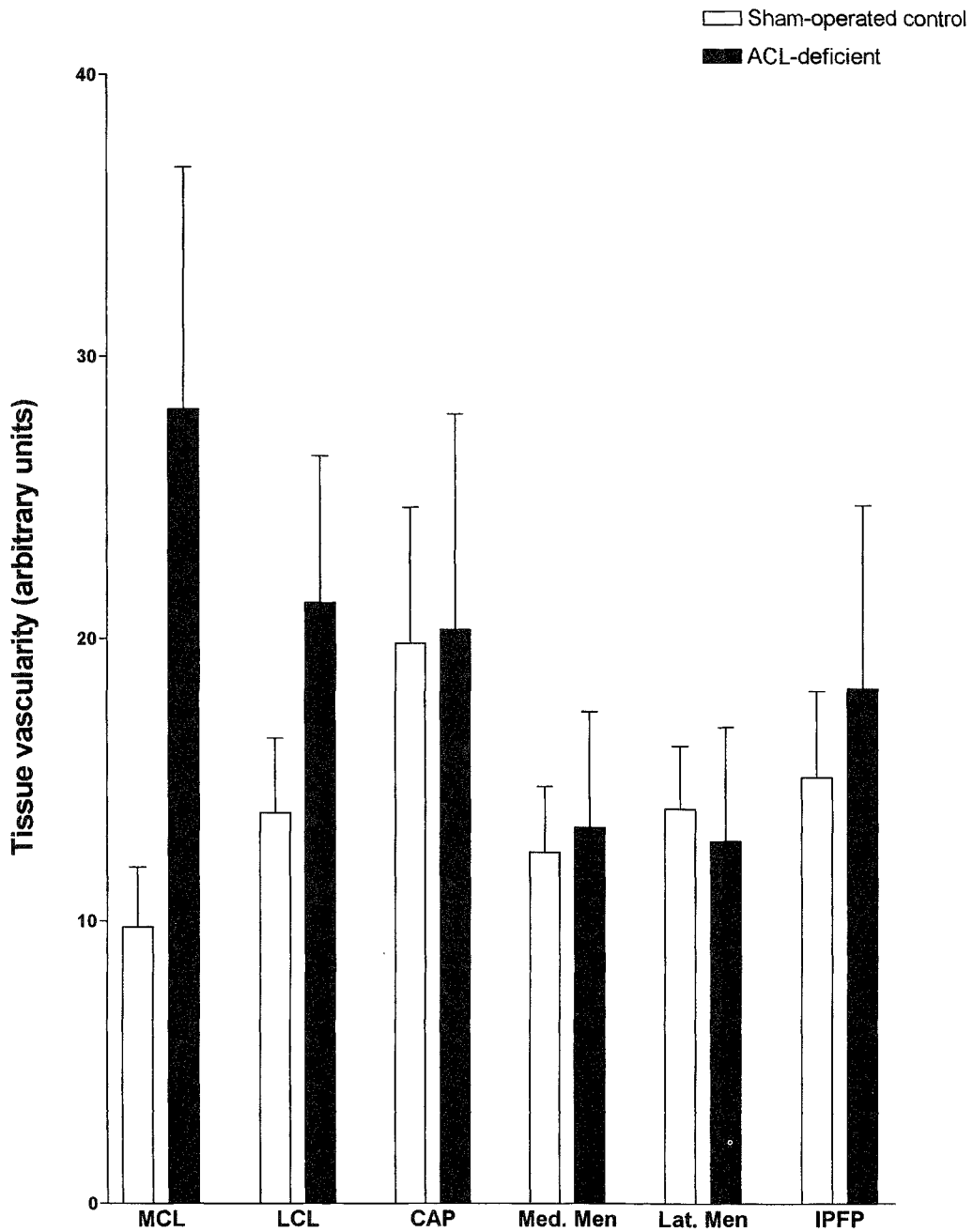


FIGURE 4. The effect of anterior cruciate ligament transection on rabbit knee joint vascularity compared to sham operated controls. The volume of vessels supplying the medial collateral ligament is significantly greater than in controls suggesting that angiogenesis has occurred in this structure as a result of microtrauma by proxy. Vascularity was not significantly altered in any of the other knee joint tissues. (Key: MCL — medial collateral ligament; LCL — lateral collateral ligament; CAP; medial capsule; Med. Men — medial meniscus; Lat. Men — lateral meniscus; IPFP — infrapatellar fat pad).

VI. FUTURE DIRECTIONS

As with all types of animal research, the study of ligament physiology and repair is only as good as the models of the human condition that they have been designed to represent. Can the reason why the MCL heals while the ACL remains latent be due to differences in biochemical composition of the two ligaments or is it related to an ineffective physiological response to injury? Even if a ligament does heal why does it never reach functional normality? The answers to these questions will only be accomplished if we advance existing models of soft tissue healing beyond where the "cut and sew" approach has failed: treatment with unique collagenases to destroy specific types of collagen; chronic administration of vasoactive or vascular remodeling drugs to alter ligament blood supply; transgenic animals which are unable to synthesize certain ligament proteins or which can promote components of abnormal morphology. These are the sorts of models we need to concentrate on if we are ever going to discover the pathways which will lead us to the Utopia of complete ligament healing.

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25 Animal Models of Tendon Repair

Donald L. Pruitt

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I. INTRODUCTION

The use of animal models has contributed greatly to our understanding of tendon physiology and biomechanics. Much of the previous investigative work has focused upon flexor tendons, probably because of the challenges clinical flexor tendon repair has presented to surgeons for many years. Flexor tendon models in dogs, chickens, rabbits and primates all have been extensively developed and described. Considerably less use has been made of animal extensor tendon models, but this should not diminish the importance of extensor tendon research. The Achilles tendon model is also well described, particularly in the rats and rabbits.

This chapter will first provide a historical review of the major animal models, describe each in more detail, and offer some thoughts on how best to choose a model for the specific question the researcher has in mind.

II. BASIC SCIENCE OF TENDON

A. TENDON HEALING

Ross¹ characterized soft tissue healing into three phases: the inflammatory, the proliferative, and the organizational or remodeling phases. In 1941, Mason and Allen² established a healing curve for divided flexor tendons in a canine model. The early stages after repair were characterized by a profound drop in tensile strength as the tendon ends softened and were held together by a gelatinous clot. They called this period the exudative stage, which lasted for the first 14 days. Thereafter, the formative stage was characterized by increased tissue organization which was directly influenced by the stresses applied to the repair site. For many years, these findings influenced clinicians to immobilize repaired flexor tendons for three weeks before beginning motion.

In 1962, Potenza³ studied tendon healing within the fibrous sheath in dogs and found no evidence of intrinsic (intratendinous) fibroblast response following tendon injury. He concluded that tendon healing depended upon extrinsic cellular ingrowth. Lindsay et al.⁴ noted fibrous adhesions attached to each point of tendon surface disruption in a chicken model. Both Potenza and Lindsay believed adhesions were an integral part of the healing process, essential for nutritional support of the healing tendon. Peacock⁵ proposed a one wound concept, which stated that healing was entirely dependent upon cells migrating into the repair site from outside sources.

In 1976, Matthews and Richards⁶ performed an experiment in rabbits which showed that the tendon injury itself was not the main stimulus for adhesion formation. Similarly, immobilization alone did not produce adhesions, but the combination of sheath injury and immobilization quickly resulted in massive adhesion formation. Lundborg and Rank⁷ showed that lacerated and repaired rabbit tendons, when implanted into the knee joint, healed by an intrinsic cellular response. Manske et al.⁸ demonstrated intrinsic healing of lacerated flexor tendons in tissue culture for rabbits, chickens, dogs and monkeys. There was some variation among species, but by 12 weeks rabbit tendons had come closest to a complete repair, followed by chicken, dog and monkey in that order.

B. TENDON NUTRITION

For a long time, tendon was considered an avascular tissue. Lundborg et al.⁹ described four vascular sources in human cadaver injection studies: longitudinal vessels extending down the tendon from the palm, vessels entering at the synovial reflections, vessels from the long and short vinculae, and vessels entering at the bony insertion. There are two bare areas between the vinculae in which no vessels could be found. It was surmised that these areas might be nourished by diffusion of nutrients. Manske et al. studied the contribution of vascular perfusion and synovial diffusion in chickens¹⁰ and primates.¹¹ They found that diffusion functions more quickly and completely than perfusion, and is a more important pathway for nutrients.

C. TENDON BIOMECHANICS

Most biomechanical studies of tendon have focused on the ultimate tensile strength and strain, both before and after repair. Tendon ultimate tensile strength has been found to range from 45 to 125 MPa,¹² depending upon the species, type of tendon, preconditioning, and animal age.¹³

Most modern suture techniques have improved purchase in the tendon such that the suture itself breaks before it pulls out of the tendon.¹⁴ Repair strength can be further enhanced with the addition of a peripheral epitendon suture.¹⁵ Gap formation can occur secondary to cyclic forces at the repair site¹⁶ and compromise the final clinical result.¹⁷ The ultimate goal of suture technique research is to develop a repair technique strong enough to allow immediate active motion.

Early attempts to optimize post operative motion protocols were largely empirical; it was hoped that early motion would decrease adhesion formation. Over the past two decades, many experimental studies have helped define the effect of motion on the tendon healing process. Using a canine

TABLE 1
Animal Models for Tendon Repair

Animal Tendon	Useful Tendons per Limb	Tendon Quality*	Cost†	Care	Best Use	Comment
Flexor						
Chicken	1–3	Thin, Flat	\$	Easy	Adhesion, Lac. and Repair	Widely Used
Rabbit	2–3	Rubbery	\$	Easy	Adhesion	Widely Used
Dog	2–4	Good	\$\$	Standard	Lac. and Repair	Widely Used
Monkey	4	Excellent	\$\$\$\$	Special	Lac. and Repair	Special Projects
Farm Animals	2–4	Variable	\$\$-\$\$\$\$	Special	Adhesion, Lac. and Repair	Special Projects
Rat, Mouse	2–4	Small	\$	Easy	Tissue Sample	
Extensor						
Chicken	1–3	Fair	\$	Easy	All	Small Size
Rabbit	2–3	Fair	\$\$	Easy	All	Small Size
Dog	3–4	Good	\$\$	Standard	All	Widely Used
Monkey	4	Good	\$\$\$\$	Special	All	Special Projects
Achilles						
Rabbit	1	Good	\$\$	Easy	All	
Rat	1	Good	\$	Easy	All	

* Tendon Quality: Compared to Human tendons

† Costs: \$=Least Expensive, \$\$\$\$= Most Expensive

model, Gelberman et al.¹⁸ found that carefully applied early protected motion allowed healing not associated with gapping at the repair site and maintenance of a smooth gliding surface. In another study comparing immobilization, delayed mobilization, and early mobilization in repaired canine flexor tendons, the load to failure of immediately mobilized tendons was twice that of immobilized tendons tested at three weeks.¹⁹ These experiments support the concept that early motion improves the quality of the biologic repair process and allows flexor tendon healing within the digital sheath while eliminating the associated adhesion formation. Hitchcock and associates²⁰ investigated the effects of immediate controlled motion on the strength of flexor tendon repairs in a chicken model and found the initial loss in strength described by Mason and Allen was not inevitable. Similar findings have been reported for extrasynovial flexor tendons in rabbits treated with continuous passive motion.²¹

III. ANIMAL AND ANIMAL MODELS SELECTIONS

Many factors enter into the decision of which animal model to select for a particular tendon research project. First one must consider the general area of tendon research being investigated. If just a small amount of tissue is required, as in *in vitro* tissue culture studies, then rats, mice or chickens may be an appropriate choice. Certain models, such as chickens, are particularly useful for investigating tendon adhesions, while others, such as canine models, are especially useful in laceration and repair studies. Nonhuman primates, such as monkeys, are often used in the final stages of evaluating prosthetic devices for possible human use.²² Table 1 summarizes selection factors for many of the major tendon models.

There are anatomic differences in tendon morphology between humans and each of the animal models. Mice and rats have very small tendons which run through a short fibroosseous canal (zone 2). Chickens have a more flat and ribbon like flexor profundus tendon compared to man. Chickens have two additional flexor tendons besides the flexor profundus, one going to the middle phalanx and one to the proximal phalanx (Figure 1). Calcification of the tendons above the hock (ankle

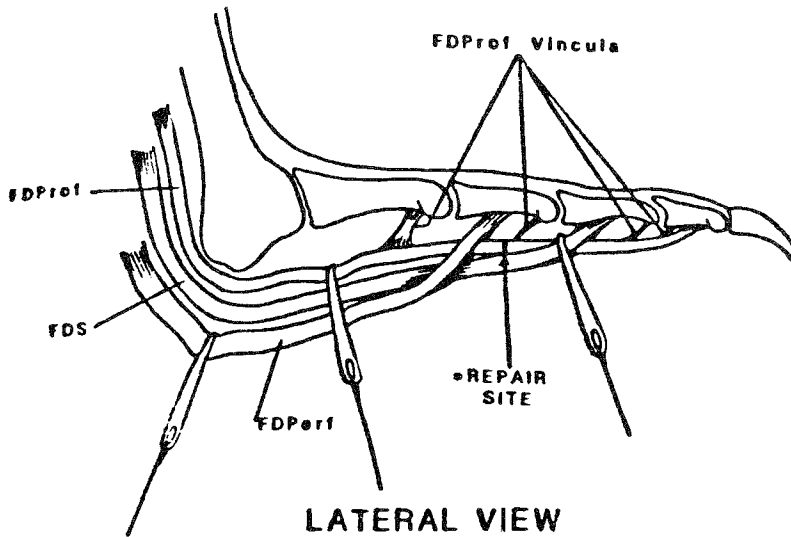


FIGURE 1. Chicken digital flexor system. One tendon inserts onto each phalanx. (From Freehan, L. M., Beauchene, J. G., *J. Hand Surg.*, 15A, 63, 1990, with permission.)

joint) is common. Dogs have a profundus tendon which is more rounded and similar in appearance to humans. Perhaps because dogs bear weight on their front paws, there is an area of fibrocartilage within the flexor tendon.²³ Monkeys, which are the most anatomically similar to humans, have a more developed ulnar side of the hand with smaller thenar muscles and flexor pollicis longus tendons.

Budgetary considerations, such as procurement and housing costs, obviously, enter into the selection process. Mice, rats and chickens are all relatively economical and can be housed in most animal care facilities. Dogs are a little more expensive to procure and house, but can usually be accommodated at most facilities. Sheep, goats, swine and other farm animals often require special housing considerations and are difficult to care for at many animal facilities. Monkeys and other nonhuman primates are the most expensive to both procure and house. Most monkeys need to be quarantined for a period of 6–12 weeks before use to prevent the spread of endemic infectious diseases which they can carry.

IV. COMMONLY USED MODELS

A. FLEXOR TENDONS

1. Chicken

The chicken model has been particularly useful in flexor tendon research. The profundus tendon travels through a long zone 2 compared to the size of the animal. This is an excellent model for studying the formation and prevention of adhesions. In addition, the tendons are usually large enough for use in laceration and repair studies.

The white leghorn chicken is the most commonly used bird. They can be obtained from the usual animal procurement agencies or directly from a farm. Chickens are rather dirty animals, and should be stored in a separate room. It is advisable to spray for mites before placing them in your cages as many animals are infested. It is also recommended that operations be performed in a room other than the usual sterile animal OR to protect the other animals.

Adult white leghorn chickens, usually hens, are used although some investigators have used cockerels.²⁴ The chickens are initially tranquilized with ketamine (10mg/kg, IM). The birds are next placed into a plastic bag which covers about two thirds of the body with holes for the feet. This helps isolate the cloaca from the operative field. The bird is then wrapped in a towel which is taped circumferentially to prevent the wings from flapping during the operation. The head is left exposed; assesment of the comb's color gives an indication of how the bird is doing. Anesthesia is obtained with pentobarbital (15 mg/kg, IM or SC).²⁵ Small additional doses of pentobarbital may be given if necessary. Foam blocks placed on each side will help keep the bird in position during surgery. A rubber tourniquet is placed around the leg above the hock. The foot is then sterilely prepped and draped. A cork board is placed beneath the sterile drapes. Sterile needles and rubber bands are used to secure the foot and hold the adjacent toes out of the way. A midline incision along the great toe is the standard surgical approach (Figure 2), although a Brunner (zig-zag) incision may also be used. Magnification is recommended because the structures are quite small. The tendons are usually repaired with a 5-0 or 6-0 core suture and a 7-0 epitenon suture. At the conclusion of the procedure, sterile dressings and either a soft or hard cast is applied. It is important to make the dressing as waterproof as possible. Antibiotics may be administered either intramuscularly or by mouth via the water supply. Even without antibiotic administration, we have seen relatively few infections despite the amount of dirt the foot is exposed to.

Numerous technical alternatives have been described for the chicken model. Some investigators have performed the procedure under local anesthesia only (with the animal's head draped to calm it down).²⁶ Others will first sedate the animal with ketamine or Nembutal and then add a local block.²⁷ Postoperative dressings have included casts with the toes in flexion,²⁸ casts with the adjacent toes in hyperextension, soft dressings allowing immediate motion,²⁹ soft dressings with a tethering splint,³⁰ (Figure (3)) and even attempts at using a continuous passive motion machine.

The chicken is an excellent model for investigating the effects of tendon injury on adhesion formation. This is most effectively performed using a partial tendon laceration. The tendon can be exposed directly, as in a laceration and repair study, or exposed indirectly by making an incision in the sheath just proximal to zone 2, pulling the tendon back into the operative field with traction on the tendon, performing a partial laceration and then allowing the tendon to retract distally back to within the tendon sheath. This technique creates a standard laceration in the tendon without injuring the adjacent sheath.³¹ A device has been described which produces a uniform 75% laceration of the tendon.³² Pharmacological agents may be injected into the tendon sheath by making a small incision over the volar portion of the distal interphalangeal joint, locating the tendon sheath and sliding a blunt tip needle or small IV catheter proximally up the sheath into zone 2.

Euthanasia is performed using an overdose of pentobarbital or intravenous potassium chloride. Good quality veins for injection are readily found in the neck. Additional veins are easily located in the wings, but are usually more fragile than neck veins.

2. Rabbit

The rabbit model has been used most for Achilles tendon and flexor tendon research. The rabbit is an excellent model for partial laceration and adhesion formation studies.³³ Rabbits are inexpensive, clean and docile animals which are easy to work with. There is a small area of zone 2 flexor tendon system within each front paw which is useful in adhesion formation studies. Rabbit tendon, however, has a more elastic or rubbery quality compared to other animals and human tendons. Previous studies have indicated that rabbit collagen is relatively more juvenile when considering collagen cross linking compared to human collagen. Thus it is harder to repair rabbit flexor tendons as cleanly as some other animal models. Despite this drawback, the model is important, especially in adhesion studies³⁴ and *in vitro* culture experiments.³⁵

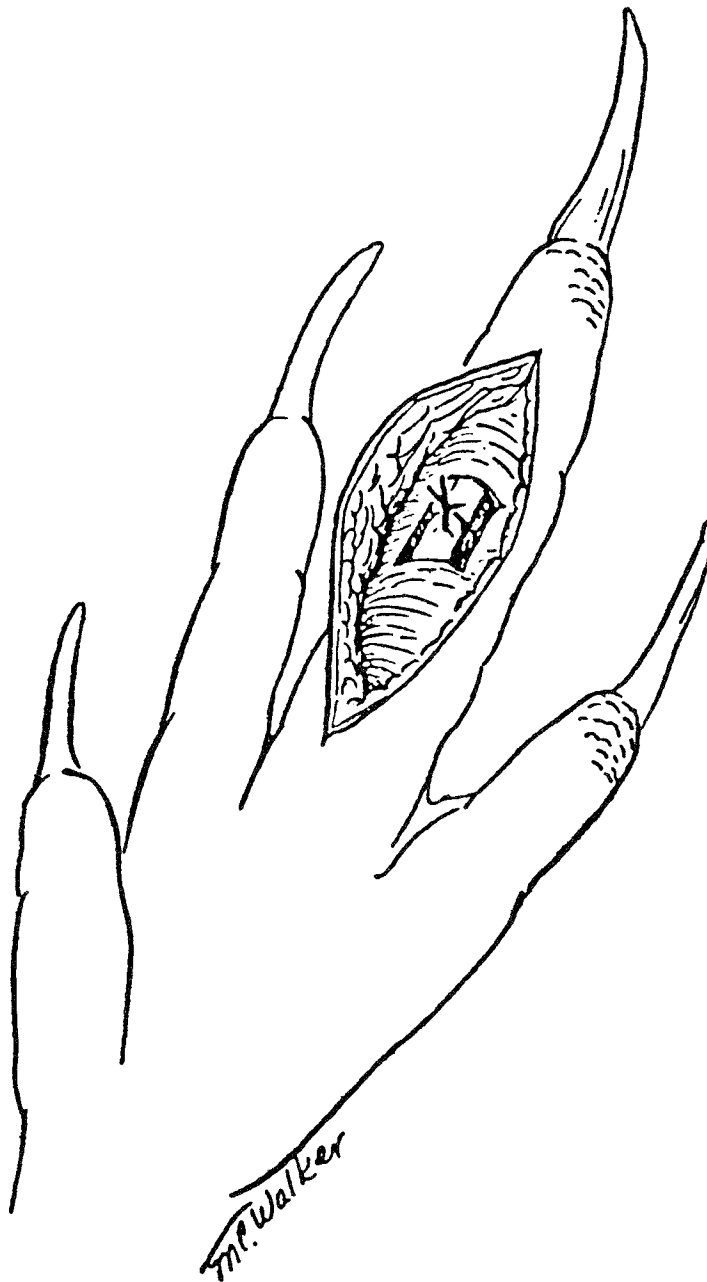


FIGURE 2. Midline approach for exposure of zone 2 of the chicken flexor tendon system. A Brunner zig-zag incision also is acceptable. (From Kessler, F. B., et al., "*J. Hand Surg.*, 11A, 241, 1986, with permission.)

Rabbits are rather timid creatures, so it is advisable to first sedate the animals with ketamine (20 mg/kg) given intramuscularly. Full anesthesia is then obtained with Nembutal or pentobarbital given intramuscularly. We have often supplemented this with a local block of 1% lidocaine. A wide Penrose drain wrapped around the limb and secured with a clamp serves as a tourniquet. The limb is shaved and then depiliated with Nair or its equivalent. The forelimb is then prepped and sterilely draped.

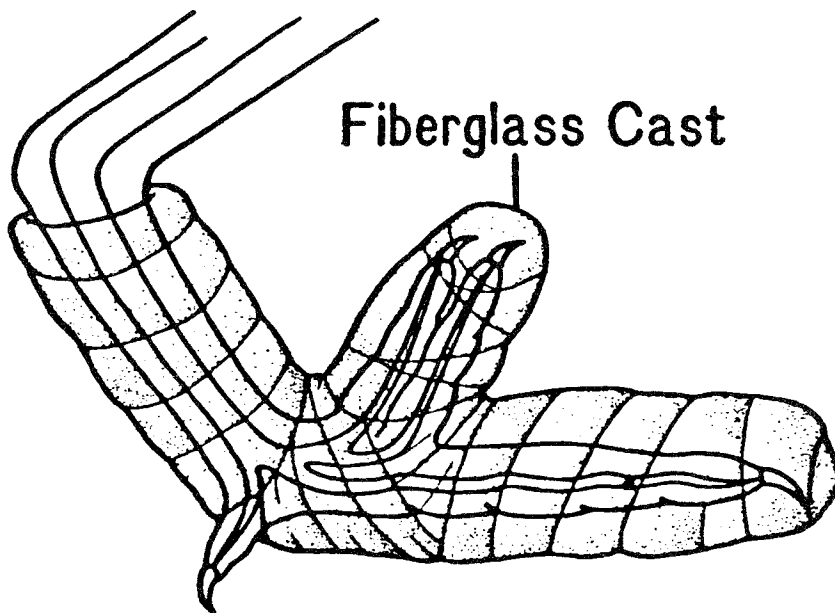


FIGURE 3. Example of the use of a cast which holds the adjacent toes in hyperextension, taking the tension off a flexor tendon repair in the chicken model. (From Hitchcock, T. F., et al., *J. Hand Surg.*, 12A, 590, 1987, with permission.)

A palmar midline or Brunner incision is used to gain access to the flexor system (Figure 4). The tendons are usually repaired with a 5-0 or 6-0 core suture and a 6-0 or 7-0 epitenon suture. Postoperatively, either a soft dressing is applied (partial laceration studies), or a fiberglass cast (laceration and repair studies) is placed above the elbow. If chewing through the cast is a problem, an Elizabethan collar may be applied. Euthanasia is usually by an overdose of pentobarbital.

3. Canine

The canine has been one of the most important models for flexor tendon research. Mason and Allen used canine wrist flexor and extensor tendons for their classic study to determine the healing rate of tendons. Dogs have also played a prominent role in studies into the formation of adhesions,³⁶ the effects of controlled motion on flexor tendon healing,³⁷ tendon suture techniques,³⁸ and many other topics.

Dogs are commonly obtained in either a conditioned or unconditioned state. Conditioned dogs have undergone veterinary evaluation and are free of worms and other parasites.

The animals are first sedated with ketamine or xylazine. Intravenous access can usually be readily gained from forepaw veins on the upper extremity. Anesthesia is induced with Nembutal or pentobarbital (IM or IV), and maintained with halothane via an endotracheal tube and a Harvard animal ventilator. The dog is placed in a lateral decubitus position with the operative limb below so that the paw pad is facing up. A pediatric tourniquet cuff usually fits well on the upper part of the limb. The extremity is taped into position, shaved and prepped and draped in a sterile manner. Administration of antibiotics, usually penicillin or cephalosporin is advisable.

After draping, the limb is exsanguinated and the tourniquet inflated to 100 mm Hg above systolic blood pressure. Zone II of the flexor tendon system lies beneath the paw pad. Exposing all four tendons requires lifting up the entire paw pad which carries a significant risk of devascularizing



FIGURE 4. Midline exposure of the rabbit flexor tendon system. The profundus tendon has been pulled proximally to expose zone 2 tendon, where a partial laceration has been made. (From Matthews, P., Richards, H., *J. Bone Joint Surg.*, 56B, 618, 1974, with permission.)

the paw pad with subsequent full thickness tissue loss. Most investigators expose only two of the flexor tendons, either the second and fifth through longitudinal incisions on either side of the paw pad, or the third and fourth tendons through a longitudinal incision in the center of the paw pad. The area is quite vascular and having an electrocautery is helpful. The tendons are usually repaired with a 4-0 or 5-0 size core suture and 6-0 epitenon sutures. Wounds are closed with 4-0 nonabsorbable sutures.

Postoperative immobilization varies from none to a shoulder spica cast, depending upon the experimental conditions. A “long arm” cast does not work well for dogs, because it moves around enough to rub full thickness sores at the elbow. Single limb shoulder spica casts or orthoses have been extensively used to protect the tendon repair.¹⁹ The area around the paw may be cut out and removed for daily passive motion exercises (Figure 5). We have often made the cast a few days before surgery and bi-valved it for easy reapplication in the operating room.

4. Primates

Monkeys and other nonhuman primates provide the closest approximation of the human flexor tendon system available in an animal model. The anatomic differences between monkey and human

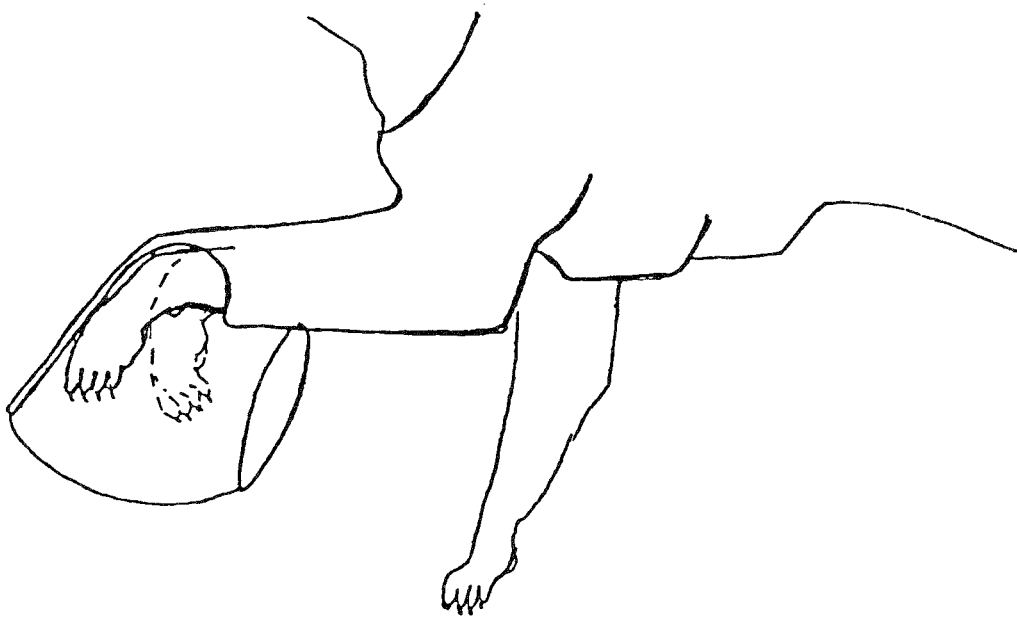


FIGURE 5. Single limb spica cast applied to a dog with the paw area cut out to allow for passive range of motion of the flexor tendons.

hands are small. In general the ulnar side of the monkey hand is more developed and the thumb and associated muscles are less developed. This probably reflects the greater importance of power grasp to the monkey rather than fine prehension.

Primates are only occasionally used in tendon research due to the high cost of both obtaining and maintaining primates. Macaca monkeys are most commonly used; they are plentiful and in no danger of extinction. Most research studies using primates are for evaluating prosthetic materials for possible human use and for projects which would not work in any other model.²²

Monkeys commonly require at least a six week quarantine period once arriving in the USA to make sure they are not carrying any diseases. They also require special cages with collapsible backs and a separate room away from other animals.

The monkeys are first tranquilized with ketamine (10 mg/kg, IM) while still in their cages. Anesthesia is then induced with sodium pentobarbital (5 mg/kg) and an airway established using an endotracheal tube. Intravenous access is also established. We typically monitor heart rate and blood pressure. Antibiotics are administered. Surgery is performed using a pneumatic tourniquet on the upper arm after shaving the hair from the operative region and a sterile prep and draping. The flexor system is approached using the same incisions as for humans: either a Brunner zig-zag or a midlateral approach. At the conclusion of surgery, sterile dressings and a fiberglass cast are usually applied. Monkeys will often chew on and pick at a cast or dressing until it is completely off, thus a rather strong dressing or splint should be applied.

5. Others

Other animals such as sheep,³⁹ cows⁴⁰ and pigs⁴¹ have been occasionally used in flexor tendon research. These models provide larger tendons to work with and have been useful in evaluating prosthetic materials. However, these animals are more expensive to procure and house at most animal facilities.

B. ACHILLES TENDON

The Achilles tendon is one of the largest extrasynovial tendons in the body. It is commonly involved in traumatic injuries, tendonitis and rupture, making it an important tendon to study in animal models. This model is also commonly used to evaluate the effects of systemic medications,⁴² exercise⁴³ and other stimuli on the musculoskeletal system. The rat⁴⁴ and the rabbit⁴⁵ are the most commonly used species, although the dog⁴⁶ is also occasionally utilized.

Rabbits are prepared for surgery in the same manner as for flexor tendons. A tourniquet is applied to the hind limb above the knee and the extremity is shaved and depilated. The Achilles tendon is exposed through a posterior midline incision. At the conclusion of surgery, a long leg cast may be applied according to the experimental protocol; a spica cast is usually not necessary.

Rats are anesthetized with sodium pentobarbital (0.5 mg/kg, intraperitoneal), and supplemented during the procedure with diluted pentobarbital (65 mg/ml) given in 0.1 ml increments. Following induction of anesthesia, the operative leg is shaved and the animal positioned on a cork board. Rubber bands looped around the legs and pinned in place with thumb tacks hold the animal in position. A Penrose drain wrapped around the leg functions as a tourniquet. The Achilles tendon is exposed via a posterior longitudinal incision.

C. EXTENSOR TENDONS

Considerably less experimental work has been carried out on the extensor tendon system compared to the flexor tendons. This may reflect the greater challenges flexor tendon repair has clinically presented to surgeons for so long. Clearly extensor tendons are different from flexors, and have become the subject of more studies in recent years.

At birth, the flexor and extensor tendons of pigs have identical mechanical properties. With growth and aging, both tendons become stronger, stiffer, less extensible and more resilient, but these changes occur to a greater degree in the higher load bearing flexor tendons compared to the flexors.⁴⁷ Recent investigations have focused on collagen fibril formation in transected tendons,⁴⁸ nutrient pathways to the tendons,⁴⁹ and the effects of early motion.⁵⁰

Models to study the extensor tendons have been described in all of the major animal models used to investigate flexor tendons. Models have included rats,⁵¹ rabbits,⁵⁰ dogs, chickens,⁵² primates and sheep. The same anesthesia, shave, prep and tourniquet placement is used as in the flexor tendon model. The paw is positioned with the dorsal surface up and a midline approach is made to the tendons. Post operative dressings will depend upon the experimental design.

D. GENERAL TENDON MODELS

Rat tail collagen has been noted to have a similar biochemical makeup and crimp pattern when compared to human tendon tissue.⁵³ Therefore, rat tail tendon is a reasonable source of tissue for ultrastructural, biochemical,⁵⁴ and occasionally biomechanical⁵⁵ studies. The tail is removed, covered in ice for 10 minutes, the ventral skin is cut for 30% of the tail length and then stripped off. This reveals the dorsal tendon bundles which can be cut free from the vertebrae for use.

Mouse tail tendon tissue may be harvested in a manner similar to the rat model for biochemical and other related experiments.

V. EVALUATION METHODS

A. MORPHOLOGICAL EVALUATION

Standard histologic methods have been used quite extensively to study tendons. For light microscopy, specimens are fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained. Haematoxylin and eosin is the most commonly used stain. Other commonly used stains

include Milligan's trichrome, Van Gieson's and Verhoeff's stains. Transmission and scanning electron microscopy are also useful techniques. Specimens for SEM may be fixed in 0.2 N Sorensen's phosphate-buffered glutaraldehyde, dehydrated in an ethanol series and dried in liquid CO₂.⁵⁶

Recent advances in molecular biology techniques permit the study of cellular processes from the beginning of specific gene expression to the synthesis of regulatory and matrix proteins. New techniques such as the polymerase chain reaction (PCR) require only small amounts of tissue allowing experiments to be carried out *in vitro*. *In situ* RNA hybridization localizes gene expression of specific proteins to individual cells within the tendon.⁵⁷ The class of regulatory proteins known as growth factors are known to be central to the regulation of wound healing and fracture repair. Their role in the regulation of tendon healing is just beginning to be defined.

B. BIOMECHANICAL EVALUATION

Standard biomechanical testing of tendons includes ultimate tensile strength and elongation to failure. The Instron and MTS mechanical testing machines are most commonly used. Difficulties measuring biomechanical properties of tendons are well recognized, especially in measuring cross sectional area and tendon elongation during testing. Clamp design flaws may allow for tendon slippage (if too loose) or breakage of tendon fibers (if too tight). Placing sandpaper around the tendon helps prevent slippage for lower force experiments. For larger tendons, uneven distribution of forces among tendon bundles may lead to inaccurate strength measurements. A photographic method for evaluating small strain fields within the tendon has been described.⁵⁸

There are two biomechanical tests commonly used to measure tendon gliding function: angular rotation and work of flexion. Angular rotation, most commonly used in dogs and chickens, uses an apparatus to measure angular rotation of the distal joints in response to a small applied load. The specimen is fastened to a metal platform at the level of the proximal phalanx and a precision potentiometer is aligned with the PIP joint. The end of the transected tendon is connected to a weight platform. In dogs, a 150 gm weight was applied for 15 seconds and the angular rotation determined.⁵⁹

The work of flexion is a quantitative representation of all of the forces that resist digital flexion. These forces include the mechanical forces which resist tendon gliding (eg. adhesions), as well as the resistance of the surrounding soft tissues related to joint movement. This parameter has been most commonly used in chickens, but can be used in other animals as well. For chickens, the feet are disarticulated at the knee and the profundus tendon to the long toe is isolated. The feet are secured to a mounting board with the toes down and a counterweight is attached to the toenail to hold the toe in full extension. The tendon end is attached to a mechanical testing machine through a load cell. The testing machine crosshead is advanced and a recording of load cell force vs. excursion is plotted on an X-Y recorder. The area beneath the curve represents the parameter work of flexion (Figure 6).⁶⁰

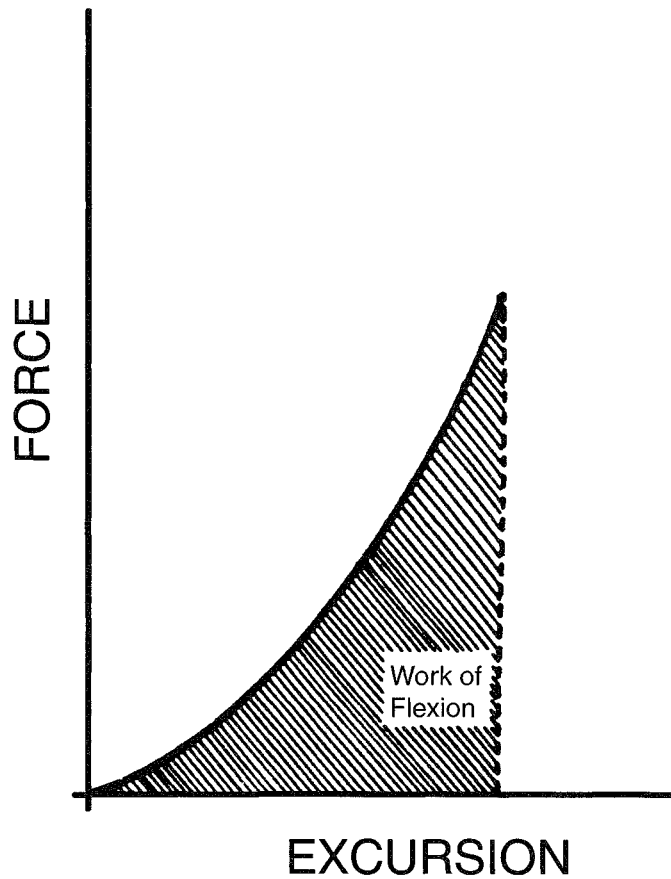


FIGURE 6. Example recording of tendon force plotted against tendon excursion. The area under the curve is the work of flexion. (From Peterson, W. W., et al., *J. Hand Surg.*, 15A, 48, 1990, with permission.)

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26 Animal Models of Ligament and Tendon Fixation

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I. INTRODUCTION

For successful transplantation or transposition of ligament and tendon, the fixation techniques are very important. The fixation must provide sufficient initial fixation strength during the postoperative period, must not interfere with the soft tissue healing, and must be biocompatible for long term use and ideally bioabsorbable or easily removable.¹

Common techniques for fixation of ligament or tendon to bone include suture, staple, screw/washer, spiked bushing, washer, or plate, bone plug or block, and interference screw (Figure 1). Several articles on comparative evaluations of different anchoring techniques have been published.^{2,3,4,5,6,7} A special tension-adjustable artificial ligament anchor has been reported recently.^{8,9} Although there is still a distance between the current version and clinical application, it brings in a new concept for ligament and tendon anchoring.

Large animals such as goats, dogs, sheep, pigs, and monkeys are common species for studies of ligament and tendon fixation. Large bone volume of these animals is the most important factor for the facilitation of bone instrumentation (fixations with screws or implants). Selected animal models from the literature for evaluating ligament or tendon fixation to bone are listed in Table 1.

Table 2 lists mechanical properties (failure load, ultimate strength, stiffness and elastic modulus) of ligament, tendon, or deep fascia of different species. Ideally, a fixation strength should exceed the requirement for normal activity on ligaments or tendons.

The healing of ligament- or tendon-bone interface has been studied histologically.¹¹ Transplanted autogenous patellar-tendon grafts undergo a process of ischemic necrosis, revascularization, proliferation, and remodeling. At one year, a transplanted graft can have the histological appearance of a normal ligament.¹¹ Kasperczyk et al.²³ defined a four stage healing process of autogenic patellar

TABLE 1
Selected Animal Models and *In Vitro* Models Used for Evaluating Ligament or Tendon Fixation to Bone

Method	Device or material	Animal species	Procedure, graft	Initial fixation strength (N)	Strength after <i>in vivo</i> study	First author, year ^{Ref.}
Suture	Suture/button	Monkey	ACLR*, PCLR†, PT‡	—	300 (1 yr.)	Clancy 1981 ¹⁰
	Stainless steel suture	Dog	ACLR, PT	—	—	Arnoczky 1982 ¹¹
Suture anchor	Suture/button	Human knee <i>in vitro</i>	ACLR, PT	248 ± 40	—	Kurosaka 1987 ¹²
	Suture over bone	Human knee <i>in vitro</i>	ACLR, iliotibial band	109 ± 11	—	Kurosaka 1987 ¹²
	Suture	Goat	Infraspinatus tendon	—	715–824 (12 weeks)	St. Pierre 1995 ¹³
	Mitek Superanchor	Human cuboid bone	Tibialis anterior tendon	223	—	Fennell 1995 ¹⁴
	Absorbable anchor	Rabbit	MCLR¶	—	—	Ono 1997 ¹⁵
Staple	Richards CC1A	Sheep	ACL MCL, artificial lig.	—	160–197 (1 yr.)	Claes 1987 ¹⁶
	Staple	Human knee <i>in vitro</i>	ACLR, PT	129 ± 16	—	Kurosaka 1987 ¹²
Screw/washer	Metal	Goat	ACLR, iliotibial band	137 ± 23	—	Kurosaka 1987 ¹²
	Metal	Goat	ACLR, fascia lata	—	200–400 (from fig.)	Holden 1988 ¹⁷
	Cortical screw	Sheep	ACLR, PE	752	1013–1233 (3 mon.)	Powers 1991 ¹⁸
	Screw/bushing	Goat	ACLR, Gore-Tex lig.	369	1380 (7 months)	Bolton 1984 ¹⁹
	2-mm cortical screw	Goat	ACLR, Kennedy LAD	364	841 (24 months)	McPherson 1985 ²⁰
	Bicortical screw	Rabbit	ACLR, bone-PT-bone	26 ± 5	51 ± 6	Ballock 1989 ²¹
	Cancellous screw	Human knee <i>in vitro</i>	ACLR, Braided PE	160	—	Gillquist 1993 ²²
	AO screw	Sheep	PCLR, bone-PT-bone	171 ± 16	708 ± 99 (2 yrs.)	Kasperczyk 1993 ²³
		Goat	ACLR, bone matrix	73 ± 9 (ligament)	474 ± 146 (1 yr.)	Jackson 1996 ²⁴

Spiked washer, plate	Plate (Synthes) UHMWPE bushing	Sheep	MCL/ACLR, artificial lig.	—	160–197 (1 yr.)	Claes 1987 ¹⁶
		Goat	ACLR, fascia lata	—	250–400 (from fig.)	Holden 1988 ¹⁷
	Cancellous screw	Pig bone	ACLR, bone-PT-bone	309	—	Paschal 1994 ²⁵
	Soft tissue plate	Tendon <i>in vitro</i>	Supraspinatus tendon	170–266	—	Gottsuer-Wolf 1994 ²⁶
	Spiked washer	Tendon <i>in vitro</i>	Supraspinatus tendon	149–514	—	Gottsuer-Wolf 1994 ²⁶
	Tendon anchor	Tendon <i>in vitro</i>	Supraspinatus tendon	399–729	—	Gottsuer-Wolf 1994 ²⁶
	AO resin or metal	Human distal femur	Fascia lata	99 ± 35, 149 ± 43	—	Straight 1994 ¹
Bone or PLA plug	Press-fit bone plug	Pig knee <i>in vitro</i>	ACLR, bone-PT-bone	463	—	Rupp 1997 ²⁷
	SR-PLA plug	Bovine knee <i>in vitro</i>	ACLR, bone-PT-bone	1100	—	Tuompo 1996 ²⁸
Interference screw	9-mm interf. screw	Human knee <i>in vitro</i>	ACLR, patellar tendon	476 ± 110	—	Kurosaka 1987 ¹²
	9-mm (DePuy)	Pig bone	ACLR, bone-PT-bone	535	—	Paschal 1994 ²⁵
	7-mm (Acufex)	Bovine bone <i>in vitro</i>	ACLR, bone-PT-bone	1358 ± 348	—	Kousa 1995 ²⁹
	7-, 9-mm interf. screw	Bovine bone <i>in vitro</i>	ACLR,	1161–1198	—	Shapiro 1995 ³⁰
	7-mm (Linvatec)	Human knee <i>in vitro</i>	ACLR, bone-PT-bone	640 ± 201	—	Pena 1996 ³¹
	Ti (Arthrex)	Pig knee <i>in vitro</i>	ACLR, bone-PT-bone	769	—	Rupp 1997 ²⁷
	AO cancel. screw	Human knee <i>in vitro</i>	ACLR, PT	208 ± 28	—	Kurosaka 1987 ¹²
	AO cancellous	Bovine bone <i>in vitro</i>	ACLR, bone-PT-bone	1081 ± 331	—	Kousa 1995 ²⁹
	SR-PLA (Biofix)	Bovine bone <i>in vitro</i>	ACLR, bone-PT-bone	1211 ± 362	—	Kousa 1995 ²⁹
Bioscrew		Same	ACLR, bone-PT-bone	418 ± 118	—	Pena 1996 ³¹
	PLA (Arthrex)	Pig knee <i>in vitro</i>	ACLR, bone-PT-bone	805	—	Rupp 1997 ²⁷
Cylindrical anchor	Titanium	Goat	ACLR, Dacron lines	—	2000–4000 (2 months)	Young 1995 ^{8,9}

* ACLR = Anterior cruciate ligament reconstruction

† PCLR = posterior cruciate ligament reconstruction

‡ PT = patellar tendon

¶ MCLR = medial collateral ligament reconstruction

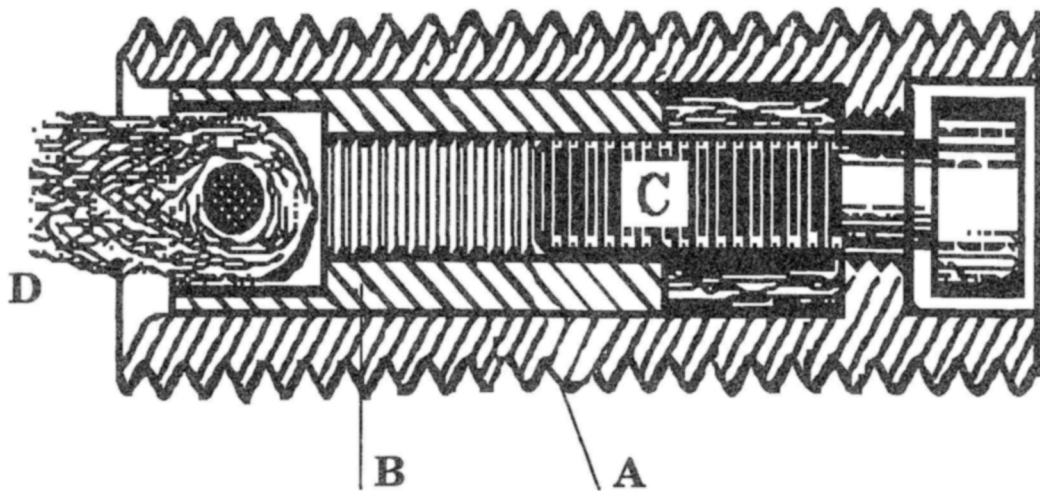


FIGURE 1. Schematic drawing of Young's artificial ligament anchor: (A) exterior cylinder, (B) internal cylinder, (C) tensioning screw, and (D) artificial ligament.

tendon graft, necrosis, revascularization, collagen formation, and remodeling. They also found that the biomechanical data were correlated with the morphological phases.

II. COMMONLY USED ANIMAL MODELS OF LIGAMENT OR TENDON FIXATION

A. SUTURE AND SUTURE ANCHOR

Suture alone and suture-over-button are common techniques for ligament fixation to bone. Common sutures include nonabsorbable suture, bioabsorbable suture, and metal wire. Suture techniques are also commonly used clinically for fixation of tendon to bone especially in hand surgery. They are used mainly for holding the tendon in place for proper healing of the tendon-bone interface. Because of the relatively low initial fixation strength, early vigorous movement is not encouraged.

Clancy et al.¹⁰ examined ACL and PCL replacements in 19 rhesus monkeys. For ACL reconstruction, patellar tendon autografts with bone attached were used. For PCL reconstruction the medial third of the patellar tendon elongated by attached portions of the patella and tibia was employed. Bone tunnels were drilled in the femur and tibia at a location which corrected for changes in ligament joint space exit location because of the size of the tunnels. Fixation was accomplished by sutures through the ligament and tied over a button. Mechanical testing results showed breaking strengths for control (medial one-third) patellar tendon specimens to be 300 N. The same setup was used to test for the grafted ligaments. Graft pull-out strengths, expressed as percentages of the strength of the medial one-third of the patellar tendon at one year, were 81% for ACL and 52% for PCL. Test results from earlier time periods gave lower numbers (e.g. ACL at two months, 34%), and it was concluded that the bone ingrowth into the tunnels was providing the increased fixation.

Arnoczky et al.¹¹ investigated the patellar tendon healing in ACL reconstruction in dogs. The proximal end of the tendon was fixed to bone by stainless steel suturing. The healing process of the graft was reported. No mechanical testing was used for evaluation.

In an *in vitro* study using human cadaver knee, Kurosaka et al.¹² investigated different fixation techniques in ACL reconstruction using bone-patellar tendon-bone grafts, iliotibial band grafts, and semitendinosus grafts. It was found that fixation strength by the suture-over-button technique was equivalent to that by staples, but was lower than that by cancellous screws and interference screws.

TABLE 2
Selected Mechanical Data of Ligament, Tendon, or Deep Fascia of Different Species

Species	Age	Materials	Failure load (Newton)	Stiffness (N/mm)	Ultimate strength (MPa)	Elastic modulus (MPa)	First author, year ^{Ref.}
Human	16-26	ACL	1730 ± 660	182 ± 56	—	—	Noyes 1976 ³²
	48-86	ACL	1734 ± 283	129 ± 39	—	—	Noyes 1976 ³²
	26 ± 6	ACL	1725 ± 269	—	38 ± 4	—	Noyes 1984 ³³
	59	ACL	559 ± 47	74 ± 3	—	—	Kurosaka 1987 ¹²
	42	ACL	2195 ± 427	306 ± 80	—	—	Rowden 1997 ³⁴
	26 ± 6	Bone-PT*-bone	2900 ± 260	—	58 ± 6	306 ± 59	Noyes 1984 ³³ & Butler 1984 ³⁵
	44	Patellar tendon	—	—	47 ± 16	—	France 1988 ³⁶
	22-35	Bone-PT-bone	2160 ± 157	242 ± 28	—	—	Woo 1991 ³⁷
	40-50	Bone-PT-bone	1503 ± 83	220 ± 24	—	—	Woo 1991 ³⁷
	60-97	Bone-PT-bone	658 ± 129	180 ± 25	—	—	Woo 1991 ³⁷
Rhesus monkey	26 ± 6	Semitendinosus	1216 ± 50	—	89 ± 5	362 ± 22	Noyes 1984 ³³ & Butler 1984 ³⁵
	26 ± 6	Gracilis	838 ± 30	—	111 ± 4	613 ± 41	Noyes 1984 ³³ & Butler 1984 ³⁵
	26 ± 6	Distal iliotibial band	769 ± 99	—	19 ± 3	—	Noyes 1984 ³³
	26 ± 6	Fascia lata	628 ± 35	—	79 ± 5	398 ± 17	Noyes 1984 ³³ & Butler 1984 ³⁵
	44	Fascia lata	—	—	32 ± 14	—	France 1988 ³⁶
	44	Achilles tendon	—	—	61 ± 26	—	France 1988 ³⁶
	Young adult	ACL	830 ± 110	194 ± 28	—	—	Noyes 1976 ³²
	Young adult	Bone-PT-bone	600 ± 132	—	—	—	Clancy 1981 ¹⁰
	Adult	ACL	1691 ± 209	453 ± 120	—	—	Powers 1991 ¹⁸
	Adult	ACL	—	259 ± 7	—	487 ± 28	Ng 1996 ³⁸
Sheep	2-year	PCL	950	130	—	—	Kasperczyk 1993 ²³
Pig	?	Toe extensor tendon	—	—	47 ± 5	980	Smith 1996 ³⁹
Rabbit	3.0 ± 0.2	ACL	—	—	57 ± 4	600 ± 50	Ishizue 1990 ⁴⁰
	3.5 ± 0.2 kg	Bone-PT-bone	About 300-400	—	—	—	Ballock 1989 ²¹
	12 months	Bone-MCL-bone	—	86 ± 1	—	—	King 1995 ⁴¹

* PT = patellar tendon

In another study, it was found that the fixation strength by a nonabsorbable suture was equivalent to interference screw and screw/washer.⁴²

St. Pierre et al.¹³ investigated the healing of tendon attachments in troughs of cancellous bone compared to direct cortical attachment. Twenty goats were subjected to a bilateral tenotomy of the infraspinatus tendon. The tendons were attached using sutures either to the cortical surface of bone or to a trough prepared in the cancellous bone. The techniques were found to provide equivalent fixation both biologically and mechanically.

Recently, different suture anchors have been developed.^{43,44} They are made of metal, nonabsorbable,¹⁴ and absorbable materials (such as the expanding suture plug [Arthrex]⁴⁵).¹⁵ No foreign body reactions were found by the studies by Barber et al.⁴⁵ and Ono et al.¹⁵ Suture anchors are playing an increasingly important role in attaching tendons or ligaments to bone.

B. STAPLE

Currently many brands of commercial fixation staples are available, such as the Richards type CC1A XSMO staple (spiked).¹⁶ Staples are convenient to use but recorded fixation strength is relatively low.

Claes et al.¹⁶ tested combined replacements of ACL and MCL of four ligament replacement materials in sheep. Some grafts were fixed to bone with a spiked staple (Richards type CC1A XSMO).

Holden et al.¹⁷ measured the strength of fascia lata autograft ACL replacements in 50 goats for periods ranging from zero to eight weeks. The objective was to compare stapled grafts to those fixed with a cancellous bone screw and spiked bushing. The latter control ACL had an average tensile failure load of 2748 N, a value which significantly exceeds that found by other investigators. At time zero, the failure force for the screw/bushing fixed specimens exceeded that for staple fixation. Other time periods yielded no significant differences in strength between the two techniques. The graft failure values were reported only as percentages of the control. At eight weeks, the value for staple fixation was 15% and the screw/bushing fixation was 9% of the control value.

Powers et al.¹⁸ conducted a study of artificial ACL replacements in goats using two tunnels each in the femur and tibia and two ligament strands to simulate the anterolateral and the posterolateral bands. Long chain polyethylene fibers were used for the ligament and staples used to fix them to bone. The increased strength obtained in the three month specimens was deemed to be the result of bone ingrowth into the tunnels providing increased resistance to pullout. Failure modes were not reported.

C. SCREW/WASHER

Cancellous and cortical screws²² with or without (commonly for artificial ligament) washers have been used.

Bolton and Bruchman¹⁹ examined the performance of PTFE (Gore-TexTM) artificial ACL replacements in 17 sheep for periods ranging from zero to 369 days. Cortical bone screws placed through eyelets built into the prosthesis were employed to fix the ligaments which were placed in bone tunnels using the "over-the top" technique. Pull-out tests were conducted, and the zero time implants yielded a mean failure strength of 1814 N. Ninety day implants with the bone screws in place yielded a failure value of 2445 N. Screws were removed from one group which averaged 218 days residence and a failure strength of 1379 N. Testing of a control group of ACL specimens yielded a failure strength of 1912 N. Fixation screws pulled out of the bone for the zero time implants, and the increased strengths observed in the experimental groups were attributed to bone growth fixation in the tunnels.

In a goat model, McPherson et al.²⁰ examined the effects of augmentation by a 6 mm PE braid of a graft ACL replacement consisting of a portion of the rectis femoris tendon, prepatellar tissue,

and the central one-third of the patellar tendon. Tensioning was secured by attaching the ligament with a bushing and cortical bone screw to the lateral surface of the femur. Tensile tests were conducted and failure typically was found to occur by pullout of the device from the tibia. The augmented ligaments were found to have a failure strength after initial implantation of 364 N. After two years, the augmented grafts had a strength of 841 N and the unaugmented grafts 528 N. These strengths were compared to a natural goat ACL quoted at 2023 N.

In a sheep model of PCL reconstruction, Kasperczyk et al.²³ investigated the healing of patellar tendon autografts. The graft was fixed to bone by screw/washer. They defined a four stage healing process of autogenic patellar tendon graft, necrosis, revascularization, collagen formation, and remodeling. They also found that the biomechanical data were correlated with the morphological phases. All ligaments failed at the ligament portion, demonstrating the effectiveness of the screw fixation.

In a goat model, Jackson et al.²⁴ attempted to improve fixation by selecting an ACL replacement material which would foster bone formation in the ligament tunnels. Demineralized bone matrix was used as the ligament and connected to a screw/washer by sutures. Six month and one year experiments were conducted in 10 goats. Seven animals were sacrificed at one year and accelerated bone formation noted in the tunnels. The mean ultimate force to failure for the reconstructed ligament at one year was 474 ± 146 N compared with the time zero strength of the matrix graft of 73 ± 9 N.

Ballock et al.²¹ reported a rabbit model of ACL reconstruction using patellar tendon autografts. They fixed the ligament graft with the spiked bushing, washer, or plate.

D. SPIKED BUSHING, WASHER, OR PLATE

Currently many brands of commercial spiked washers or plates are available, such as the Synthes type 65.00.11 soft tissue fixation plate,¹⁶ the AO polyacetal resin spiked washer and AO soft tissue fixation plate.¹

Holden et al. studied the effect of a spiked bushing (with 5-mm diam. shaft) on the fixation of fascia lata grafts for ACL reconstruction in a goat model. The results showed that at eight weeks, the strength of the graft was 9% of the control value, compared to 15% achieved by staple fixation.¹⁷

Claes et al.¹⁶ tested combined replacements of ACL and MCL of four ligament replacement materials in 30 sheep for one year. Carbon fiber (Lafil), polydioxanone strand, dacron, and a bovine tendon xenograft were employed. The combined replacement technique employed three bone tunnels and a continuous (ACL-MCL) replacement. Both prosthesis ends were anchored on the lateral surface of the femur using either a staple or a spiked fixation plate with screw (Table 1). Tensile tests were conducted for MCL and ACL separately with the staples or fixation plate removed. No ligaments fractured during the tensile tests and failure occurred by pulling the ligament out of the bone tunnel. However, no effects of the fixation techniques on the strength of the fixation and ligament healing were found.

Gottsauner-Wolf et al.²⁶ tested different methods of fixation of tendons to metal prostheses, including soft tissue fixation plate (Synthes), spiked polyacetal washer (Synthes), and a new enhanced tendon anchor (ETA), a device with spikes designed to interlock both prosthesis and tendon and held in place by two screws. Attachments were made both to the bone blocks of tendon/bone combinations and directly to tendons. Canine supraspinatus tendon was used and 60 "motion units" were tested. The ETA required the largest force to remove, but none of the methods were as strong as the control intact muscle-tendon unit. The use of tendon with attached bone block significantly increased the fixation strength.

E. BONE OR ABSORBABLE PLUG

Bone plugs or blocks either separately used with artificial ligaments⁴⁶ or connected with biological grafts²⁷ are commonly used clinically for reconstruction of the ACL. The fixation strength of press-fit bone plugs was found to be lower than that found with interference screws.²⁷ The fixation of bone blocks in the bone tunnel is often reinforced by using an interference screw or sutures tying to a screw/washer on the surface of the bone.

Ligament fixation using a self-reinforced PLA expansion plug was reported by Tuompo et al.²⁸ in a bovine bone model. The maximum tensile strength of the SR-PLA plug was above 1100 N and the initial strength of the absorbable plug is strong enough for clinical use.

F. INTERFERENCE SCREW

The recent trend toward early motion and aggressive postoperative rehabilitation emphasizes the need for secure initial graft fixation before bony incorporation of the graft.^{32,47} Kurosaka et al.¹² in an *in vitro* study demonstrated that the 9-mm interference screw (DePuy) had superior initial fixation strength over a 6.5-mm cancellous screw. One shortcoming of interference screws is their difficulty to remove which makes revision surgery difficult sometimes. Then, bioabsorbable interference screws, such as Bioscrew, appeared.^{28,31} The initial fixation strength has been reported to be lower³¹ or equivalent to metal ones.^{27,29,48}

Paschal et al.²⁵ compared postfixation (tying to cancellous bone screw) to interference screw fixation in 20 frozen/thawed pig knees using bone-patellar tendon-bone ACL replacement grafts. Displacement of the graft in the bone tunnel by a force of 110 N was measured as well as the load required to pull out the graft from femurs and tibias separately. Displacements were highest in the postfixation and higher in tibias. The strength of the postfixation for femurs was 274 N while for interference it was 543 N. For tibias, the postfixation strength was 343 N and the interference screw strength 527 N. All grafts failed at the point of fixation.

In another study using pig knees, Rupp et al.²⁷ compared press fitting of the bone block in a bone-patellar tendon-bone graft to the use of a biodegradable (polylactic acid) interference screw. Titanium interference screw fixation served as a control. Pull-out force to failure was measured. The biodegradable screw fixation yielded a load of 805 N and the titanium screw 769 N. The press fit yielded a lower load of only 463 N. All specimens failed at the attachment site.

Kousa et al.²⁹ also examined interference screw fixation in harvested knees. Bone-patellar tendon-bone ACL replacement grafts were placed in frozen/thawed bovine knees. Interference screws, cancellous bone screws, and fibrillated PLA screws were used. Tensile failure strengths ranged from 1081 N to 1358 N with no statistically significant differences.

Shapiro et al.³⁰ investigated the screw size on the pullout strength of *in vitro* ACL reconstruction using bovine knee. The results showed there was no significant difference between 7- and 9-mm interference screws. However, in another study, it was found that failed bone plug fixed by 7-mm screw could be refixed successfully with a 9-mm screw.⁴⁹ Jomha et al.⁵⁰ found that there was a significant weakening of fixation for screw–bone plug angle equal or more than 20 degrees.

G. YOUNG'S LIGAMENT ANCHOR

Young and An reported a new adjustable screw anchor to secure the artificial ACL prosthesis to the femur and tibia (Figure 1).^{8,9} Fixation was provided by screw threads on the exterior surface of a hollow cylinder which was placed in the bone tunnels created in the femoral condyle and tibial plateau. The artificial ACL was attached to a sliding portion inside the threaded cylinder, which was adjusted for tension by means of a screw accessed from outside the exterior bone surfaces. Push-out tests of anchors which had functioned for two months in goats gave values of approximately 2000–4000 N.

III. EVALUATION METHODS

Histomorphological analysis is the basic evaluation method for the effect of ligament or tendon fixation to bone.^{11,16} The ligament- or tendon-bone interfaces are harvested *en bloc*. The tissue blocks can be embedded in paraffin (for decalcified tissues without fixation devices) or plastic media (for tissues containing fixation devices).

Mechanical testing (tensile test) is the most important method for evaluating the function of the ligament- or tendon-bone interface. Initial¹ and long term mechanical strength of the ligament and tendon fixation should be tested (Table (2) (see Chapter 10). When tensile strength is measured, one has to be aware of where the failure occurs — the fixation device or the ligament itself either at the ligament–device interface or the ligament body. Normally, the ultimate strength of a ligament–device–bone assembly represents the maximum load the fixation device can hold. The ultimate strength of a fixation device or anchor can be tested with an “unbreakable ligament” (stronger than the device holding strength) in a tensile mode or using a pushout test as was the case for Young’s ligament anchor.^{30,31}

Biochemical evaluation has been used for examining DNA content, content and types of collagens, PGs or GAG synthesis and content in the repair tissues⁵¹ (see Chapter 6). MRI also has been used for evaluating ligament healing in the bone tunnel.²²

IV. CONCLUDING REMARKS

To summarize, repaired or substituted ligaments and tendons are provided with some sort of mechanical fixation in the form of screws, plates, or sutures to allow healing to take place which will provide the long term survival of the repair. The values obtained by investigators indicate that very long time periods are necessary before bone ingrowth can support either grafts or alloplastic replacements. Initial fixation strength is essential for early movement and rehabilitation programs. The major causes of artificial ligament failure include stretching, loosening and breaking. Future research should focus on initial fixation strength, good long-term bony incorporation, bioabsorbable or easily removable anchors, reduction of artificial ligament stretching, and breakage.

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Part VII

Animal Models of Spinal Conditions



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27 Animal Models of Spinal Instability and Spinal Fusion

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I. INTRODUCTION

Spinal fusion (arthrodesis) is the standard surgical treatment for instability of the spine and has been utilized clinically since 1911. Albee experimentally examined the concept of intersegmental spinal fusion by conducting macroscopic and microscopic studies of posterior spinous process fusions in canines.¹ Since that time, live animal “models” have been routinely used to evaluate individual factors influencing the outcome of spinal surgery. They provide reproducible and quantifiable information which is often difficult or impossible to obtain from human subjects, cadaveric human or animal models, or simulations such as finite element analysis.

Animal models of the spine are, in many ways, distinct from those used to examine other interventions to the skeletal system. Often, spinal column realignment and stabilization with arthrodesis in a mechanically advantageous arrangement may be the preferred treatment of a spinal disorder. In contrast to arthrodesis of the major joints of the extremities, selective elimination of motion across pathologic vertebral motion segments does not necessarily result in significant functional incapacity for the organism as a whole. Fusions of destabilized vertebral segments are among the most frequently modeled treatments to the musculoskeletal system.

The first section of this chapter will review general concepts of spinal instability and stabilization, and provide a summary of the animal models that have been used previously and the applications for which they are useful. The second section will focus on animal models that have been used to evaluate novel osteoinductive growth factors or implantable, fusion enhancing biomaterials. Models that enable examination of systemic factors influencing the fusion process will also be discussed.

II. MODELS OF INSTABILITY AND INTERNAL FIXATION

Animal models for *ex vivo* and *in vivo* simulation of spinal instability and stability, and of *in vivo* spinal fusion are presented in this section.

A. BIOMECHANICAL INSTABILITY

Although poorly understood and difficult to assess clinically, spinal instability is considered one of the primary causes of chronic spine-related pain and progressive neurologic deficit. It is speculated that dysmorphic and excessive intervertebral motions cause abnormal deformations of ligaments, apophyseal joint capsules, annular fibers and vertebral endplates such that the nociceptors in these respective tissues trigger pain responses.² In addition, the increased excursion of the adjacent neural elements is thought to cause compression or stretching of these structures resulting in radicular symptoms and deficits. The precise relationship between dysmorphic motion and clinical symptoms has not been determined. However, accurate assessment of instability is deemed critical to appropriate management of these disorders.

Panjabi's revised definition of instability is based upon the size of the neutral zone, "that part of the range of physiological intervertebral motion, measured from the neutral position, within which the spinal motion is produced with a minimal internal resistance."³ The neutral zone may increase with injury and incompetence of constraining soft tissue and bony elements, and degeneration and incompetence of the constraining apophyseal joints. It may decrease with paraspinal muscular strengthening, osteophyte formation, internal fixation, and osseous fusion.

A change in the neutral zone may be more sensitive than a change in the corresponding range-of-motion for determining instability. Neutral zone measurements incorporate muscular and peripheral soft tissue contribution to spinal instability whereas range-of-motion measures may not. Historically, biomechanical studies have focused on the mechanics of passive components and, consequently, range-of-motion measures.⁴ Thus, implications of active components such as muscular forces in both normal and pathologic conditions are not well understood.

Recently, a simulation of lumbar instability, examining passive and active stabilization components, was developed in an *in vivo* porcine model.^{5,6} Sequential surgical injuries in the L3-L4 intersegment were created and sagittal kinematics were measured. Greater axial translation occurred following injury to the disk and annulus; greater sagittal rotation and shear translation occurred following graded injuries to the facet joint. Interestingly, muscular stimulation following each of these selected injuries increased sagittal rotation and shear translation while decreasing axial translation. Paradoxically increasing range-of-motion, muscular stimulation reduced abrupt kinematic behavior in the neutral region, reducing the neutral zone. This methodology provides one of the few experimental demonstrations of the neutral zone theory of instability. However, anatomic dissimilarities between the human and porcine posterior spinal elements and muscular attachment points impose limitations to this model.

B. EX VIVO MODELS OF BIOMECHANICAL INSTABILITY AND FIXATION

Several animal models have traditionally been used to evaluate the passive components of spinal stability, examples as shown in Table 1.⁷⁻¹⁴ Among *ex vivo* cadaveric models, the bovine spine has

TABLE 1
Ex Vivo Biomechanical Evaluation of Animal Spine Models

Species	Spine Specimen, Preparation	Conditions, Evaluation	First Author, year ^{Ref.}
Dog	Rib cage-thoracic spine complexes T5-T9	Resection of costovertebral joints; destruction of the rib cage	Oda 1996 ⁷
Calf	Anterior and middle column defect L2-L5	Anterior fixation with intervertebral body graft after discectomy and endplate excision of L3-L4; anterior fixation only	An 1995 ⁸
Pig and Human	Destabilization with fixation	TSRH;* bone graft; bone graft + TSRH; BAK†; BAK + TSRH; normal spine	Brodke 1997 ⁹
Pig	Interspinous lig. and bone specimens	Collagen lig. interrupted with progressive disruption	Dickey 1996 ¹⁰
	L3 corpectomy with/fixation	Anterior strut graft with spinal nail fixation	Dawson 1996 ¹¹
	C3-4 discectomy and dissection of posterior longitudinal lig.	Hydroxyapatite with anterior plating; ICBG‡ with anterior plating; ICBG	Takahashi 1997 ¹²
Sheep	T8-L7 bisegmental specimens	Mechanical testing: extension-flexion, axial rotation, lateral bending	Wilke 1997 ¹³
Dolphin	Vertebral column	T1-T8 three segment specimens External loads applied to intervertebral segments	Long 1997 ¹⁴

* TSRH = Texas Scottish Rite Hospital fixation device

† BAK = intervertebral fixation device (Spine-Tech, Minneapolis, MN)

‡ ICBG = iliac crest bone graft

been the most widely used and validated for the thoracic and lumbar regions. Calf spine specimens, especially, are relatively homoscedastic in bone strength and mineral density and may be preferred over human specimens for mechanically testing particular instrumentation constructs.

Wilke et al. determined range-of-motion, neutral zone, and stiffness properties of thoracolumbar bovine spines (T6-L6) under pure moment loading in flexion and extension, axial left/right rotation, and right/left lateral bending. Similarities in axial rotation and lateral bending were noted between bovine and human cadaveric spines. The bovine spine was suggested to be in a limited way a substitute for the human spine.¹³

The pull-out strength of bone screws placed in bovine vertebral pedicles correlated significantly with the bone mineral density of vertebral bodies. Since the bovine vertebral bodies had significantly higher and less variable mineral density (146 mg/ml, $p < 0.05$) than humans, failures consistently occurred within the implant construct rather than the bone implant interface. The bovine spine is ideal for testing the performance of spinal fixation constructs since there is less interspecimen variability compared with the human cadaveric spine.¹⁵

Zdeblick et al. have performed L3 corpectomies in calf spines to test stabilization afforded by several anterior fixation devices. In torsion, they found the Kaneda anterior fixation device to be the stiffest, while in axial compression and flexion-extension modes, the Kaneda device and the Texas Scottish Rite Hospital (TSRH) fixation device were the stiffest.^{16,17} Earlier, L3 corpectomies in calf spines were used to examine both anterior and posterior fixation devices under mechanical, non-destructive cyclical testing in axial compression, rotation, and flexion.¹⁸ Again the Kaneda device, with fixation one level above and one level below the corpectomy site, was the stiffest anterior fixation system. The Cotrel-Dubousset and Steffee transpedicular fixation systems, incorporating vertebral segments two levels above and below the corpectomy site, were the stiffest posterior fixation systems. Other investigators have used the bovine spine as a model to evaluate instrumentation constructs in the treatment of traumatic or scoliotic conditions.^{19,20,21,22}

The bovine model is also an excellent choice for studying the biomechanics of lumbosacral fixation.²³ Although the bovine spine anatomically has smaller vertebral width, six lumbar vertebrae, less lordosis, and larger transverse processes, the range-of-motion at the usually sagittally hypermobile lumbosacral junction was quite similar to the human spine.

The bovine spine was used to biomechanically evaluate the efficacy of posterior instrumentation systems for stabilization of artificial isthmic spondylolisthesis of the lumbosacral joint and found that transpedicular fixation was superior to older distraction constructs.²⁴

The bovine spine has also recently been used to evaluate the performance of disc-replacing intervertebral fixation devices using either the anterior or posterior approach for lumbar intervertebral fusion (ALIF, PLIF). *Ex vivo*, an intact bovine lumbar spine was used to demonstrate that PLIF procedures performed with structural iliac crest bone graft (ICBG) were less stiff than intact spines.⁹ The use of transpedicular fixation increased initial stiffness significantly (2.5 times the intact spine), and the use of a threaded, fenestrated, cylindrical, and hollow intervertebral fixation device (BAK device; Spine-Tech, Minneapolis, MN) without transpedicular instrumentation, also increased initial stiffness (2 times the intact spine). Other intervertebral fixation devices such as the NOVUS™ fusion device (Sofamor-Danek, Memphis; TN) have been evaluated in the bovine spine with similar results.^{25,26,27,28}

Ex vivo animal models of the cervical spine are less frequently used than those of the thoracic and lumbar spines. A bovine model of flexion-distraction cervical injury was used to study stabilization methods in a constrained, nonrepetitive loading environment.²⁹ Recently, a porcine model was used to evaluate three methods of cervical spine stabilization.³⁰ In contrast to previous animal and human cadaveric instability models, the specimens were destabilized with a one-level cervical corpectomy and tested under unconstrained and repetitive loading following instrumentation. This investigation demonstrated that anterior grafting combined with posterior lateral mass plating achieved maximum stability. The conclusion was that despite a difference in facet orientation, the

porcine spine was ideal for simulation of human cervical spine fixation due to the close size match and otherwise similar geometry.

C. *IN VIVO* MODELS OF SPINAL INSTABILITY AND FIXATION

In vivo animal models of destabilization and fixation are necessary to examine the effects of skeletal repair and intersegmental fusion on intersegmental instability (Table 2).

An *in vivo* animal model of both anterior and posterior column instability was developed to study the effects of bone arthrodesis and remodeling after instrumentation.⁵⁰⁻⁵² A destabilizing lesion was created at the L5-L6 intersegment by sectioning both the disc annulus and the posterior elements in adult beagles. Animals that underwent instrumentation had a higher probability of achieving fusion than those that did not. Furthermore, among all successful fusions, those that had instrumentation were stiffer in torsion, axial compression, and flexion following removal. It was also shown, in this model, that osteoporosis was linearly related to the rigidity of the implants in one of the first demonstrations of device related osteoporosis.

In order to simulate an unstable burst fracture, laminectomy, partial facetectomy, and corpectomy of the L5 vertebral segment were performed in canines. Comparison of anterior strut graft with instrumented fixation and without fixation yielded a higher fusion rate in the former. Among the fusions, the spines that had been instrumented were significantly stiffer to torsion.¹⁷

Rates of fusion may vary considerably within a quadrupedal animal depending upon the segmental level that is being treated.⁴⁶ Specifically, posterior intertransverse process fusion (PLSF) without instrumentation was achieved across the interlumbar motion segments of the sheep lumbar spine. In contrast, fusion almost never occurred at the lumbosacral junction with the same treatment. This finding was attributed to increased motion at the lumbosacral junction compared to the intralumbar levels. Similarly, the findings at lumbosacral junction in canines may be attributed to the kinematic behavior at this level.⁵³ These findings along with studies on kinematic behavior of quadrupedal animals indicate that forces, loads, and treatment results are not equal across segmental levels.^{36,54,38,55,56,57,58}

Load-sharing capacity of spinal instrumentation and the posterolateral fusion mass has also been studied in the sheep model following posterolateral fixation and bone grafting with transpedicular fixation.⁴⁸ Stability with instrumentation followed by stability after removal of the instrumentation was measured. Fixation provided anterior and middle spinal column support which enabled the spinal intersegments to better resist eccentric loads in the sagittal plane.

Increased motion at spinal levels adjacent to spinal fusion is well documented in clinical literature. *In vivo* motion data from the L2-L3 intervertebral segment were measured before and 12 weeks after posterior spinal instrumentation from L3-L7 in the canine.³⁹ Vertebral rotations in the coronal plane and excursion of the facet joints increased significantly after the caudal instrumentation. Tissue responses to changes in abnormal joint motion may be investigated using this canine model.

The influence of spinal implants and instrumentation for anterior cervical fusion has been examined in the caprine model.^{59,17} A three level anterior cervical discectomy without posterior destabilizing lesions was performed. Several different implant conditions were tested in the discectomy sites including a sham condition. Each discectomy site in a single animal was treated with the same condition.⁶⁰ A higher rate of fusion was observed with autograft than with allograft. In a second study, the addition of anterior cervical instrumentation with the autograft did not increase the fusion rate.⁶¹

D. SUMMARY OF SPINAL INSTABILITY AND INTERNAL FIXATION

Ex vivo animal cadaveric models have generally been used to test instrumentation constructs without the confounding influences of quality of the bone and biologic factors associated with

TABLE 2
***In Vivo* Animal Models Used for the Biomechanical Evaluation of Spinal Instability**

Animal	Type of Procedure, Level	Conditions and Evaluations	Time	1st Author, Year ^{Ref.}
Rabbits	Injury to facets L1–2, L2–3	Low activity vs. high activity; bilateral facet excision L2–3; unilateral facet excision L1–2, L2–3; facets exposed only	2 weeks, 1, 6, 12 months 6 weeks	Stokes 1989 ³¹
	Posterior fusion of interspace L3–L6	ICBG* + instrumentation with or without decortication; exposure + instrumentation; exposure only		Ishikawa 1994 ³²
Dogs	Posterior midline fusion L4–L5	Bone graft + wire fixation+ PMMA; bone graft + wire fixation; bone graft	8 weeks	Feighan 1995 ³³
	Distraction hooks and rods L2, L3	Instrumented; removal of instrumentation	2–6 months	Kahanovitz 1984 ³⁴
	Steinman pins, transpedicular L2, L3	Standing, walking and moving from sitting to walking, turning, and moving from a 4-leg stance to hind leg position. documentation of motion	N/A	Wood 1992 ³⁵
	Strain gage, L2–3 facet joints	Loading during static and dynamic activities	3 days	Butterman 1992 ³⁶
	Posterior facet fusion L3–L4, L4–L5	6.35 mm diam. rods retained and removed 12 weeks; 4.76 mm diam. rods retained & removed after 12 weeks; not operated (control)	12 weeks	Craven 1994 ³⁷
	L3–L7, Steinman pin fixation	<i>In vivo</i> vs. <i>in vitro</i> motion at segment L2–L3	1, 12 weeks	Dekutoski 1994 ³⁸
	Posterior fusion L3–L7, Steinman pins	<i>In vivo</i> motion data collected while animal walked on a treadmill.	1, 12 weeks	Schendel 1995 ³⁹
	Posterior fusion of L1–L5	ICBG* + screws at L1, L3, L5 + rods; ICBG	3, 6 months	Kioschos 1996 ⁴⁰
	Intervertebral body fusion	Dx cervical spondylomyelopathy with plug for distraction-stabilization	?	Dixon 1996 ⁴¹
	Laminar & PLSF†; fixation L2, L3, L4	Macroporous ceramic, autograft (laminar vs. intertransverse sites)	9 months	Delectin 1997 ⁴²
Pigs	Bilateral laminectomy and facetectomy L5–L6, with fusion	Fusion alone vs. fusion with down sized VSP plates	24 weeks	Edwards 1997 ⁴³
	Surgical injury to L3–L4 segment	Disc injury; facet joints removed; facet and transverse processes removed	N/A	Kaigle 1995 ⁶
	Chronic lesion model L3–L4	Anulus injury; facet capsule injury; facet joint slit; facet joint wedge; sham	3 months	Kaigle 1997 ⁵
	Discectomy and fusion C2–C3, C3–C4, C4–C5, C5–C6	HA; tricortical ICBG	6, 12, 24 weeks	Pintar 1994 ⁴⁴
Sheep	Posterior fusion L3–L5	3,2, 4,8, 6,4 cm rod, pedicle screws + ICBG; non rodged spines	16 weeks	Johnston 1995 ⁴⁵
	Posterior laminar fusion/Steinman pins	ICBG across decorticated lamina of L3, L4, L5, L6, sacrum; L6–S1 fusion with decortication, fixation + ICBG; nonoperated	4–43 weeks	Nagel 1991 ⁴⁶
	PLSF, L3–L4, L4–5	Autograft + decortication + C-D† instrumentation; decortication + C-D instrumentation; C-D instrumentation; non operated	1 year	Guigui 1994 ⁴⁷
	Posterior fusion of L1–L5	ICBG + transpedicular screw fixation; ICBG removal of fixation	16 weeks	Kotani 1996 ⁴⁸
	PLSF, L2–L3, L4–5	ICBG + fixation; one level with transpedicular screw fixation.	8, 16 weeks	Kanayama 1997 ⁴⁹
	ALIF‡, L4–L5 or L5–L6	ICBG + Ti cage; autograft dowel; sham; nonoperated	24 weeks	Sandhu 1996 ²⁵

* ICBG = iliac crest bone graft

† C-D = Cotrel-Dubouset instrumentation

‡ PLSF = Posterolateral intertransverse process fusion

§ ALIF = anterior lumbar interbody fusion

osseous fusion. In contrast, *in vivo* animal models mainly incorporate the effects of biologic factors such as skeletal repair and remodeling mechanisms and mechanical factors such as the *in vivo* kinematic behavior of specific vertebral segments.

The bovine spine has been the most widely used among animal models for examining the effects of destabilization and internal fixation of the thoracic and lumbar spines *ex vivo*. This has largely been due to the general anatomic similarities and the increased and highly consistent mineral density of the bovine spine compared to the human spine. Both the bovine and porcine models have been used similarly to examine the cervical spine *ex vivo*. Although neither of these models anatomically mimics the human spine with regard to cervical facet joint orientation, the relative similarities of size and geometry were sufficient to warrant their use.

The canine, sheep, and porcine models have been most commonly used to examine both anterior and posterior instability and fixation in the thoracic and lumbar spines *in vivo*. Canines have been particularly popular because of ease of post-operative care in enclosed institutional environments and the ability to treat these spines with conventional forms of internal fixation devices. The alpine goat model is one of the few established for *in vivo* study of the cervical spine primarily due to the ease of approaching the cervical spine surgically, the similarity of vertebral size and geometry with the human, the larger sized intervertebral discs, and the relative ease of post-operative management and evaluation. Finally, the porcine model has been successfully used to examine the active muscular component of spinal column stability, and to examine such stability from the standpoint of the neutral zone.

III. ANIMAL MODELS OF SPINAL FUSION AND BIOLOGY

Established animal models in spinal fusion studies, their specific advantages and limitations, and the significance of the data derived from each are reviewed. Animal models evaluating osteoinductive growth factors are presented in Table 3, and those evaluating biomaterials are presented in Table 4.

Biologic factors influence the success or failure of spinal fusion. The impact of biologic “tools” such as osteoinductive growth factors, which stimulate the biologic processes of skeletal repair, must also be examined rigorously in animal and procedural models wherein successful fusion is difficult to achieve. In order to examine these factors carefully, selective animal models for the study of biologic factors of spinal fusion must be used and interpreted appropriately.

The requirements of an animal model for the study of biologic processes differ from those examining mechanical factors in that the former considers simulation of human geometric relationships and loading characteristics to be relevant, but not critical. The primary endpoint is usually the formation of an osseous fusion mass which connects adjacent vertebral motion segments and exists in space previously occupied by soft tissue. Although local kinematics and mechanical forces may influence success or failure of the fusion attempt, other factors, such as fusion technique, fusion location, evolutionary complexity of the animal, size of the animal,¹¹² bone architecture,¹¹³ and contents of the bone graft or bone graft substitute may take precedence in determining the outcome of treatment. For example, it is common in lower animals that facet and interlaminar fusions occur simply by surgical exposure and periosteal stripping of the posterior spinal elements.⁵⁴ In contrast, the nonhuman primate spine, like the human, often fails to achieve either facet, interlaminar, or intertransverse process fusion despite meticulous decortication and abundant autogenous bone graft along the transverse processes.¹⁴⁹ The speed at which osseous fusion takes place also varies considerably between species and is influenced by the location of the fusion and the type of graft.

Historically, several animal models have been used to examine biologic variables which affect spinal fusion. Rats, rabbits, dogs, guinea pigs, sheep, goats, and nonhuman primates among others, have been treated with facet, interlaminar, intertransverse process, spinous process, anterior interbody and posterior interbody fusions. Study designs have included single level fusions, multiple adjacent level fusions with distinct or similar implants at each level, multiple separated level fusions with distinct or similar implants at each level, and single level fusions with distinct implants on either side.

TABLE 3
Animal Models Employed in the Evaluation of rhBMPs (rhBMP-2, rhBMP-7, etc.)

Animal	Fusion Procedure	Experimental Conditions, Implant	Time (wks)	1st Author, Year ^{Ref.}
Rats	PLSF,* L4-L5	rhBMP-2 + collagen; ICBG; collagen only; decortication only	4	Dawson, 1998 ⁹⁵
Rabbits	PLSF,* L5-L6	rhBMP-2 + collagen; rhBMP-2 + collagen + ICBG†; rhBMP-2 + ICBG; or collagen	5	Schimandle 1995 ⁶²
	PLSF, L5-L6	bBPF-DBM-collagen; DBM alone; autograft alone	5	Boden 1995 ⁶³
	PLSF, L5-L6	rhBMP-2 + collagen; ICBG	4	Hollinger 1996 ⁶⁴
	Lateral intertransverse process L4-L5	rhBMP-2 + collagen; collagen	10	Boden 1996 ⁶⁵
	PLSF, L5-L6	bBP + biocoral + collagen; biocoral alone;	5	Boden 1997 ⁶⁶
	PLSF, L5-L6 with ICBG	Fusion masses separated into outer and central zones, RNA extracted RT/PCR done for BMP	10	Boden 1997 ⁶⁷
Dogs	Laminar medial transverse process T6-T7, T8-T9, T10-T11, T12-T13	Decortication + autograft (spinous process); BMP/PLA° or PLA; Decortication only	3, 24	Lovell 1989 ⁶⁸
	Facet spinous process fusion T13-L1, L2-L3, L4-L5, L6-L7	rhOP-1§ (rhBMP-7) + collagen; collagen; match sticks autograft; implant	6, 26	Cook 1994 ⁶⁹
	Posterior segmental fusion, L1-L2, L3-L4, L5-L6	Superficial decortication of lamina instrumented: dual plates, rhBMP-2/PLGA;^ PLGA alone; proximal humerus autograft	12	Muschler 1994 ⁴⁶
	PLSF, L4-5 with decortication	rhBMP-2 + open-pore PLA; open-pore PLA; morselized ICBG	12, 32	Sandhu 1995 ⁷⁰
	PLSF, L4-5 with decortication	rhBMP-2 + open-pore PLA; open-pore PLA; morselized ICBG	12	Sandhu 1996 ⁷¹
	PLSF, L4-5	rhBMP-2 + open-pore PLA with or without decortication	12	Sandhu 1997 ⁷²
	PLSF, L4-5 with no decortication	rhBMP-2 + collagen; rhBMP-2 + open-pore PLA;	12	Sandhu 1996 ⁷³
	PLSF, L4-5 with decortication, bilateral paraspinal approach	rhBMP-2 + collagen; rhBMP-2 + open-pore PLA collagen; corticocancellous rib graft	12	David 1996, ⁷⁴ 1996 ⁷⁵
	Spinous processes, lamina, facet fusion, T13-L1, L4-L5, L2-L3, L6-L7	rhBMP-2 + collagen + ICBG; collagen + ICBG + cohesive paste material; ICBG; no implant	6, 12	Sheehan 1996 ⁷⁶
	PLSF, T13-L1, L2-L3, L4-L5, L6-L7	rhBMP-2/collagen + morselized ICBG; ICBG + collagen	16	Helm 1997 ⁷⁷
	PLSF, T13-L1, L2-L3, L4-L5, L6-L7	rhBMP-2/collagen + ICBG collagen + morselized ICBG autologous morselized ICBG; no implant	12	Li 1996 ⁷⁸
	PLSF, L4-L5	54, 215, or 860 mg rhBMP-2 + collagen; 215 mg rhBMP-2 + open-pore PLA; collagen; ICBG	12	Sandhu 1996 ⁷³
	Bilateral laminectomy, L5	rhBMP-2/collagen placed on the dura; autogenous bone from laminectomy site placed on dura	12	Kwiatkowski 1997 ⁷⁹
	PLSF, L1-L2, L3-L4, L5-L6	ICBG alone; ICBG + rhBMP-2, ICBG + collagen “sandwich” + rhBMP-2, ICBG + collagen morsels + rhBMP-2, ICBG + PLGA sponge sandwich + rhBMP-2, and ICBG + open-pore PLA morsels + rhBMP-2	8	Fischgrund 1997 ⁸⁰

Goats	AIF, # C1-C2, C2-C3, C3-C4 AIF, C2-C3, C5-C6 AIF, C2-C3, C4-C5 AIF, C2-C3, C4-C5	Ti BAK + rhBMP-2; HA ∞ -coated BAK + local reamed graft; BAK + local reamed graft 50/50 porous HA-TCP ^o (30, 50, or 70% porosities); 50/50 HA-TCP; autograft rhBMP-2 + HA-TCP + plate; HA-TCP + Ti cage; rhBMP-2 + HA-TCP + Ti cage; HA-TCP + plate; rhBMP-2 + HA-TCP rhBMP-2 + 50% porous 50/50 HA-TCP; HA-TCP; rhBMP-2 calcium carbonate (Bicoral); Bicoral alone	12 12, 24 16 12, 16	Zdeblick 1995 ⁸¹ Toth 1995 ⁸² Toth 1997 ⁸³ An 1997 ⁸⁴
	Anterior cervical fusion C2-C3, C3-C4, C4-C5 Anterior cervical disectomy, fusion, C2-C3, C3-C4, C4-C5	rhBMP-2/collagen sponge + plate fixation; saline/collagen sponge + plate fixation; ICBG + plate fixation rhBMP-2/collagen sponge + Ti lordotic cages; local bone graft + Ti lordotic cages; rhBMP-2/collagen sponge + porous Ta \S intervertebral cages; local bone graft + Ta cage Autograft dowel; autograft + Ti cage; rhBMP-2/collagen sponge/Ti cage	11 12, 24	Bolesta 1997 ⁸⁵ Moore 1997 ⁸⁶
Sheep	ALIF, # L4-L5 or L5-L6 ALIF, T5-T6, T7-T8, T9-T10 ALIF, L4-L5 or L5-L6	BAK + rhBMP-7/collagen sponge; BAK + ICBG; tricortical ICBG, video assisted thorascopy rhBMP-2 with porous Ta cylinder; porous Ta cylinder	24 16 24	Sandhu 1996 ⁸⁶ Cunningham 1996 ⁸⁷ Sandhu 1996 ⁸⁷
Rhesus	PLSF, L4-L5	bBP/DBM (groups); DBM alone	12	Boden 1996 ⁸⁸
Monkeys	Laminectomy & lateral intertransverse fusion, L4-L5 ALIF, L7-S1	rhBMP-2+collagen; rhBMP-2 in HA-TCP (60:40); collagen, report of technique only rhBMP-2/collagen with rhBMP-2 soaked freeze dried cortical dowel; ICBG with freeze dried allograft cylinder rhBMP-2/collagen/TIF \S ; collagen/TIF; laparoscopic exposure	N/A 24 24	Boden 1996, ⁸⁵ 1997 ⁸⁹ Boden 1997 ⁸⁹ Hecht 1997 ⁹⁰
	ALIF, L-S1		24	Boden 1997 ⁹¹

* PLSF = posterior intertransverse process fusion
† ICBG = iliac crest bone graft
‡ bBP = bovine osteoinductive bone protein
§ PLA = polylactic acid
^ PLGA = polylactide-co-glycolide acid
§ rhOP = recombinant human osteogenic protein, same as rhBMP-7
AIF = anterior interbody fusion
 ∞ HA = hydroxyapatite
¶ Ta = tantalum
£ TIF = Tapered Interbody Fusion Device
¢ ALIF = anterior lumbar interbody fusion
◊ TCP = tricalcium phosphate

TABLE 4
Animal Models Used for the Evaluation of Spinal Fusion Implants

Animal	Fusion Procedure(s)	Experimental conditions, implants	Time	1st Author, Year ^{Ref.}
Mice	PLSF* C1-C2	Autologous tail; ICBG; † transgenic ICBG; syngeneic donor	4 weeks	Rhee, 1998 ⁹²
Guinea Pigs	L-S (guinea pigs), L4-L5 (dogs)	Dowel graft from iliac crest; H-graft; posterior ICBG (guinea pigs); upper thoracic spinous process graft (dog)	6-72 weeks	Thomas 1975 ⁹³
Rats	PLSF; last three lumbar vertebrae, last 3 thoracic vertebrae	DBM one side + decortication; DBM both sides, spinous processes, interspinous lig., capsule, cartilage of facet joints removed + decortication; bone graft, spinous processes, interspinous lig., capsule, and cartilage of facet joints removed + decortication	3-60 weeks	Guizzardi 1991 ⁹⁴
Rabbits	PLSF; L4-5 Spinous interspace fusion Spinous interspace fusion, T3-T4, T7-T8	Decortication only; ICBG DBM; allogenic deep frozen cortical bone; auto cancellous bone Bone marrow + DBM; DBM; bone marrow	12 weeks ? weeks 2-20 weeks	Dawson 1998 ⁹⁵ Oikarinen 1982 ⁹⁶ Lindholm 1988 ⁹⁷
	Intervertebral fusion T12-L1, L1-L2 PLSF; L5-L6	HA microcrystals; rhTGF-β1 + HA microcrystals; vs. powdered T12 rib graft Healos matrix; Healos with autogenous bone marrow; Healos with heparinized autogenous bone marrow; ICBG	12 weeks 8 weeks	MacMillan 1997 ⁹⁸ Tay 1997 ⁹⁹
Dogs	AIF‡ PLSF; L4-L5 PLSF; L5-L6 Interfacet interlaminar fusion L1-L2, L3-L4, L5-L6 Facet joint fusion T7-T8 Unilateral facet fusion, T13-L7 Laminar and facet fusion Interfacet interlaminar fusion	Bicoral; Kiel; autogenous rib graft Autologous bone marrow + morselized ICBG; clotted blood + morselized ICBG 2.5 cm ³ per side of ICBG Collagen + ceramic, 15-30 kDa proteins; ICBG; no graft Percutaneous approach: HA cancellous bone plugs DBM with freeze dried allograft from canine cancellous bone; ICBG PLA; PLA + marrow; ICBG; decortication only Bone marrow + collagen ceramic (50:50); autogenous cancellous bone, collagen ceramic (50:50); autogenous bone Calcium carbonate + fixation; calcium carbonate; tricortical ICBG HA; Biphasic (60:40) HA/TCP; Calcium carbonate; ICBG 40% TCP + 60% HA contact with lamina, facets, transverse processes; 40% TCP + 60% HA in lateral vertebral grooves; ICBG laminar, transverse processes	12 weeks 12 weeks 6 weeks 12 weeks 6 mo. 12 weeks 24 weeks ? weeks 8 weeks 8 weeks 9 mo.	Tho 1996 ¹⁰⁰ Curylo 1997 ¹⁰¹ Toribatake 1997 ¹⁰² Muschler 1993 ⁵⁵ Stein 1993 ¹⁰³ Cook 1995 ¹⁰⁴ Callear 1995 ⁵⁴ Muschler 1996 ⁵⁷ Fuller 1996 ¹⁰⁵ Emery 1996 ¹⁰⁶ Delecrin 1997 ¹²

Goats	Anterior discectomy and fusion, C2-C3, C3-C4, C4-C5, Anterior discectomy and fusion, C2-C3, C3-C4, C4-C5 PLIF§, L4-L5 ALIF¶, L7-S1	Frozen allograft; autogenous tricortical bone graft; disc excision only; 10/21 fused with tircortical autograft	12 weeks	Zdeblick 1992 ⁶⁰
Pigs	PLSF, L3-L4, L4-5 ALIF, L1-L2, L3-L4, L5-L6	ProOsteon (Interpore) + cervical plate; ProOsteon (Interpore) + no plate; porous HA; autograft + anterior plate; autograft; no fusion Carbon fiber-reinforced polymer implant-ICBG; allograft bone Open procedure vs. laproscopic procedure implants: ICBG with Ti cage (MOSS) Porous coral or biphasic ceramic + decortication + Correl-Dubousset instrumentation DBM; replamineform coral; plaster of Paris; autogenous pelvis bone; autogenous or frozen allograft; Ti cage alone	12 weeks 6 mo. 12 weeks 1 yr. 12 weeks	Zdeblick 1994 ⁶¹ Brantigan 1994 ¹⁰⁷ Riley 1997 ¹⁰⁸ Guigui 1994 ⁴⁷ Nicodemus 1997 ¹⁰⁹
Sheep	Anterior discectomy and fusion C3-C4, C5-C6 Anterior fusion, cervical	Autograft + Cervi-Lok; autograft + Ti cage (BAK-C); ICBG; discectomy alone; not operated Unthreaded bone basket + autograft	12 weeks 8-15 yrs	Goldstein 1997 ¹¹⁰ Bagby 1988 ^{11a} , 1997 ^{11b}

* PLSF = Posterolateral intertransverse process fusion

† ICBG = iliac crest bone graft

‡ AIF = anterior intervertebral fusion

§ PLIF = posterior lumbar intervertebral fusion

¶ ALIF = anterior lumbar intervertebral fusion

A. POSTERIOR FUSION ANIMAL MODELS

Canines have historically been the most commonly used animal models to examine factors that influence the spinal fusion process. Albee performed 13 thoracic and lumbar fusions in canines using autograft, allograft ulna, and xenograft ulna to first define the fusion process and the contributions of different graft materials.¹ Hurley, in a classic study in 1959, used the canine to define the role of the paraspinal soft tissues in osteogenesis.¹¹⁴ Posterior fusions in 37 dogs were performed with either a filter barrier to cells and fluid from the paraspinal tissues, a filter barrier to cells only, or no filter at all. The absence of a filter, or the use of a filter to cells only allowed a solid fusion at L5-L6. However, the use of a filter to cells *and* fluid consistently resulted in nonunions. These findings were among the earliest evidence that the surrounding paraspinal soft tissues provided a source of nutrition and possibly diffusible growth factors necessary for skeletal repair to transpire.

Bone graft enhancers or substitutes have been evaluated in the canine model. Callewart et al. used a lumbar (T13-L7), four level alternating facet and interlaminar anthrodesis in the beagle model.⁵⁴ By 24 weeks, facet joints fused spontaneously (77% fusion) simply with decortication of the bone surface of the facets and lamina. Treatment with morselized ICBG resulted in a higher facet fusion rate (100%) compared to decortication alone, or a polylactic acid polymer containing autogenous bone marrow (33%). In dogs, Lovell et al. used an alternating four level, thoracic interlaminar fusion procedure to perform the first investigation of a purified bovine bone morphogenetic protein (bBMP) extract as a bone graft enhancer.⁶⁸ Levels that were implanted with bBMP combined with autograft had a higher rate of fusion (71%) radiographically and histologically as compared to the best control sites (29%). In earlier animal studies, BMP had been identified as an osteoinductive morphogen capable of inducing *de novo* bone in extraskeletal sites.¹¹⁵

A high fusion rate was achieved with another bBMP extract (15–30 kDa) and with ICBG controls in alternating multilevel lumbar interlaminar fusion performed in canines.⁵⁵ Subsequently, a recombinantly produced human BMP (rhBMP-2) was compared to autograft yielding high rates of fusion in both.⁵⁶ Another recombinant BMP growth factor (rhBMP-7) was examined via multilevel facet lumbar fusion procedure in which four unilateral fusions in alternate levels of the lumbar spine, T13-L7, were done in canines.⁶⁹ Although a high fusion rate was noted with the use of the growth factor, all sites implanted with autograft fused in this facet and laminar fusion model albeit at a slower rate (12 weeks with growth factor and 26 weeks with autograft). Alternating, multilevel (T13-L7) spinous process, lamina, and facet fusions were completed in canines. rhBMP-2 was used as a bone graft enhancer and demonstrated a higher rate of spinous process, lamina, facet, fusion with the growth factor as well as a greater volume and greater stiffness in the fusion mass compared to control conditions.⁷⁶ The criticism of each of these studies, is that facets and lamina fuse too readily in canines to enable valid comparison with experimental conditions.

Sandhu et al also used the canine model to examine the performance of rhBMP-2 but refined the fusion technique to increase the difficulty of achieving fusion.⁷⁰ Rather than facet and laminar fusions, in a series of experiments single level L4-L5 bilateral intertransverse process fusions (PLSF) were performed. Successful fusion was defined as solid osseous bridging between the transverse processes. Dogs implanted with morselized ICBG along the transverse processes in this single level L4-L5 fusion model failed to achieve successful fusion within three months, but did so after six months. However, all animals (100%) that had been treated with rhBMP-2, ranging in dose from 58 mg to 2300 mg, went on to successful fusion within the three month time period.⁷¹ A further study, using a similar single level fusion procedure only *without decorticating* the posterior elements, demonstrated that an 89% fusion rate could be achieved with rhBMP-2.⁷²

A bilateral paraspinal approach followed by transverse process decortication was used to also examine rhBMP-2 in a fibrillar collagen delivery vehicle for PLSF.^{74,75} They reported a 100% fusion rate with the growth factor and a 33% fusion rate with autograft.

Schimandle and Boden have argued that the rabbit model is more cost-effective, easier to manage, allows adequate ICBG harvest, and simulates the human condition as well as the larger

quadrupedal animals do.^{116,117} They used a single level (L5-L6) PLSF technique in rabbits to investigate rhBMP-2 with a collagen carrier and found 100% fusion with the growth factor and 42% fusion with autograft within four weeks of implantation.⁶² Since the fusion rate observed in the control condition was similar to that seen in human studies of uninstrumented PLSF, this animal model combined with surgical technique was suggested as an appropriate simulation of the human condition.

Subsequently, Feiertag et al. modified the above model to study nonunion of spinal arthrodesis.¹¹⁸ Of 35 rabbits that had undergone L5-L6 PLSF with ICBG, 23 were subjected to a "lifting" protocol to induce motion at the fusion site. The lifted animals exhibited a 13% rate of fusion compared to a 50% rate of fusion in control animals that had not been lifted. A relatively high 17% mortality rate secondary to anesthetic and surgical complication was noted in this study and was consistent with other reports using this model.

Lower mammalian models have been criticized with regard to biologic investigations since the biology may differ sufficiently and, therefore, findings may not be extrapolated to higher animals such as nonhuman primates and humans. Aspenberg et al. have asserted that known osteoinductive implants such as demineralized bone matrix (DBM) or BMP reliably induced extraskelatal bone formation in rodents but did not reliably do so in higher animals and in primates.¹¹⁹ DBM implants augmented with rhBMP-2 *did* induce intramuscular bone in squirrel monkeys but the quantity of bone was less than that seen in lower animals. They presume that the higher the evolutionary complexity of the species, the fewer BMP-2 receptor expressing cells there are available in the surrounding extraskelatal tissues. For this reason, studies of bone inducing growth factors that have used higher animal models such as nonhuman primates have been considered even more relevant to the human condition.

Boden et al has used PLSF in rhesus monkeys to further explore implants previously examined in the rabbit model.⁸⁸ Recently, they demonstrated successful dose-dependent L4-L5 intertransverse process spinal fusions in this model using a purified bovine osteoinductive bone protein extract. Although the number of animals tested was limited, those implanted with higher doses of the osteoinductive implant achieved fusion whereas those implanted with lower doses or the carrier only did not fuse. Lately, Boden has explored a less invasive approach to the fusion site.⁹¹

B. ANTERIOR FUSION ANIMAL MODELS

Anterior interventions on the spinal column have different requirements than posterior interventions. Animal models employed for the study of anterior spinal fusion generally are larger and capable of safely undergoing transperitoneal, retroperitoneal, transthoracic, or anterior cervical approaches to the spine. Since fusion entails obliteration of the disc space and osseous bridging across the intervertebral gap, anatomic relationships that mimic the human condition including relatively large disc spaces and parallel vertebral endplates are preferable. Most commonly they are goat, sheep, pig, and rhesus monkey models and less commonly the canine.

An anterior intervertebral fusion, T7-T8, was performed in beagles to examine calcium carbonate ceramics in a load bearing area of the spine.¹⁰⁵ Discectomy was followed by decortication of the adjacent endplates. The implant was placed into the discectomy site. A lower fusion rate was found with the bone substitute (5%) than with tricortical ICBG (75%). ICBG implants were superior to hydroxyapatite, tricalcium phosphate, and calcium carbonate ceramic implants in another study employing the same surgical procedure and animal model.¹⁰⁶

Lumbar intervertebral fusions were performed on Spanish goats. Spines implanted with the carbon-fiber reinforced polymer implant containing autogenous bone graft achieved a quicker and more reliable fusion than those with ethylene oxide-sterilized allograft bone.¹⁰⁷

A retroperitoneal approach was used to perform anterior interbody fusions (ALIF), L5-L6, in sheep. A cylindrical, fenestrated titanium cage was either packed with ICBG or filled with the growth factor, rhBMP-2, on a collagen sponge and implanted in the fusion site. Greater ingrowth of *de novo* bone through the cage and a higher fusion rate was found with rhBMP-2 compared to

autograft (100% vs 33%).^{25,26} A subsequent study using this same surgical technique and animal model found a higher histologic fusion rate for a threaded porous tantalum cylinder implanted with rhBMP-2/collagen than without it (100% vs 17%).²⁷

Rhesus monkeys were implanted with cylindrical allograft bone dowels combined with either rhBMP-2 on a collagen sponge or ICBG using the same ALIF, L7-S1, surgical open approach.⁹⁰ The primates implanted with rhBMP-2 achieved fusion as early as six weeks, a finding not observed in animals implanted with ICBG. More remarkably, the allograft dowels with rhBMP-2 underwent complete resorption and substitution by 12 weeks suggesting an acceleration of the remodeling process by the addition of rhBMP-2.

Cervical fusion techniques and implants have primarily been evaluated in the caprine and sheep models. An adjacent three level anterior cervical discectomy, decortication of the endplates, and fusion of the subaxial cervical spine performed in alpine goats to compare tricortical ICBG, tricortical allograft, and no graft.^{60,61} Segments implanted with ICBG had a higher rate of fusion than those implanted with either allograft or no graft. Addition of the internal fixation did not increase the fusion rates.⁶¹ Subsequently, coral hydroxyapatite bone substitute, autogenous tricortical bone graft, and fresh-frozen tricortical allograft with and without internal fixation were examined using the same three level surgical fusion procedure in the caprine model.⁶¹ A significant rate of collapse was noted with the ceramic but some evidence of early creeping substitution was present. In this study, internal fixation prevented implant extrusion and improved the fusion rate in the group receiving the ceramic. Recently, the alpine goat model was employed to evaluate titanium intervertebral cages designed for the cervical spine. There was a 100% fusion rate for sites implanted with rhBMP-2 in collagen compared to 86% fusion rate with autograft.⁸¹

Two level (C2-C3, C4-C5) anterior discectomy and fusion procedure with ceramics as bone substitutes placed in the discectomy site was also examined in the caprine model. Sixty seven percent of hydroxyapatite-tricalcium phosphate (HA-TCP) implanted sites fused compared to 50% of the autograft implanted sites. By the time of explantation, 50% of the implanted ceramics fractured. The rate of fracture was unrelated to the porosity of the ceramic.⁸² The fusion rate and fracture rate did not change with the addition of rhBMP-2 to HA-TCP or with an alternate ceramic (bicoral calcium carbonate ceramic).⁸³

The three level cervical fusion procedure in the caprine model was recently used again. The effect of rhBMP-2 soaked collagen sponge inserted into the discectomy site with adjacent vertebrae fixed with an anterior plate was compared to tricortical ICBG without internal fixation.⁸⁵ Fusion rates were not statistically different possibly due to the small sample size. Both titanium and tantalum intervertebral fusion cages with and without rhBMP-2 were evaluated in the above model, with the greatest mechanical stiffness following fusion noted with the rhBMP-2-implanted tantalum cages.⁸⁶

C. SURGICAL APPROACHES AND TECHNIQUES

Although posterior lateral intertransverse process fusion (PLSF) is the most common surgical technique performed in clinical practice, historically, facet and interlaminar fusions have been the most often performed techniques in animal models. There is sufficient evidence that success rates of these distinct methods vary considerably and that results of one method cannot be extrapolated to predict results of another. For example, facet and interlaminar fusion following decortication and autogenous bone grafting is easily achieved in the beagle whereas intertransverse process fusion is far more difficult in the same animal model.^{54,70,73}

There are several variations of the posterior intertransverse process fusion surgical technique. A bilateral paraspinous approach is used by some investigators ('lateral transverse process fusion')⁷⁵ whereas a midline incision is used by others.^{70,65} Variations also exist in the amount and anatomical sites of decortication executed in similar fusion attempts.^{32, 72}

A minimally invasive modification to the common open surgical approach of intertransverse process fusion procedure was developed in the rabbit and rhesus monkey model.⁶⁵ rhBMP-2 carried by a fibrillar collagen vehicle was implanted in four rhesus monkeys.

Laparoscopic, endoscopic, dorsal, and open approaches have been used to accomplish an anterior interbody fusion implanting cages filled with growth factors in sheep and rhesus monkeys.

The sheep model was first used to examine multilevel anterior interbody fusions, T5-T10, using video-assisted endoscopic technique (VATS) vs. the open technique to implant the BAK device either with ICBG, tricortical ICBG, or in combination with an anterior fixation plate.⁸⁷ Laparoscopic ALIF was performed in the rhesus monkey using titanium intervertebral fusion cages which contained either rhBMP-2 with a collagen carrier or the collagen carrier alone.⁹¹ All primates implanted with rhBMP-2 achieved successful fusion whereas none of the animals implanted with carrier alone did.

D. VERTEBRAL SEGMENTS

The location of a fusion procedure has significant bearing on the outcome. In the canine and sheep models, the fusion rate at the lumbosacral junction, which is likely influenced by the local kinematics, is far lower than that of adjacent levels.^{46,53,54} Although multilevel fusions in the same animal represent a cost-effective design methodology, valid comparisons between the treated vertebral segments require counterbalancing the scheme of treatment conditions so that treatments are equally influenced by the inherent motion at each of the treatment levels.

To internally control for both the location of the fusion level and the idiosyncratic variability of the animal, a within-animal experimental design is appealing. However, a within-animal design of comparative treatments at the same level (i.e. experimental treatment on the left side and control treatment on the right side of L4-L5) uses a methodology that is flawed. The effect of one of the treatments on the motion segment will invariably influence the outcome of the other treatment. If one side is fused, the other is more likely to also be fused independent of any treatment effect.

E. QUADRUPEDAL ANIMAL MODELS

There are two significant considerations in the lower mammalian spine that further distinguish these models from the human condition. First, in quadrupedal models, the loads applied to the ambulating spine are quite different from those applied on the ambulating human spine (bipedal). Specifically, posterior elements of the cervical and lumbar portions of the spine are under tension in the quadruped as opposed to compression in the bipedal human. Since skeletal repair is influenced by these distinct mechanical forces, the biologic simulation of the human condition is, to some extent, undermined.

Second, the control condition in these models, to which all experimental conditions are compared, requires the harvest and transplantation of the current clinical standard of graft material, autogenous cortico-cancellous bone from the iliac crest (ICBG). This control is selected simply because it is the technical standard in clinical practice. However, the quality and quantity of retrievable ileum may be quite different in the animal models compared to the human. For example, in the lower mammals, the relative proportion of cortical to cancellous bone is higher than that of humans, and often the transplanted graft consists largely of cortical strips with sparse cancellous bone. Theoretically, the control condition in these models should not be considered a precise reflection of the standard of clinical practice.

F. METHODS OF EVALUATION

From a mechanical standpoint, the intent of an intervertebral spinal fusion is to create an osseous mass of bone consolidating portions of adjacent vertebral segments such that the intervertebral neutral zone is substantially reduced to acceptable levels. Noninvasive methods employed to determine success of fusion in the animal model are similar to those used clinically. Interval plain radiographs and even computed tomography scans can be useful in determining the quantity of *de novo* bone formation, the continuity between the fusion mass and the elements of the spinal column, and dynamic instability of the fusion segment by using positional radiographs.

The advantage of the animal model is that additional methods can be used for more detailed evaluation. Typically, the spines are explanted at predetermined time points and evaluated with gross manipulation, nondestructive and destructive mechanical testing, and microscopic analysis. Manual palpitation and gross manipulation of the fusion site addresses the question of fusion (no motion) or no fusion (motion). Mechanical testing can objectively measure stiffness by applying loads and measuring the corresponding displacements. The most useful data, however, is often gleaned from histologic analysis which determines not only *de novo* bone formation, but also describes the extent of remodeling and host reaction to the intervention. Manual and mechanical testing, as well as histologic analysis provide data on performance of alternative implants in animal models. Such analysis, of course, is not available clinically.

Given the same animal model and spinal fusion procedure, various time intervals for observation have been employed. Comparison among results from different studies may be difficult because of distinct end points of evaluation.

G. INHIBITORS TO SPINAL FUSION

The intertransverse process spinal fusion procedure in the rabbit model has been used to study the effects of nicotine exposure,¹²⁰ critical period for removal of nicotine,¹²¹ and anti-inflammatories. Other factors and substances for the promotion and inhibition of spinal fusion may be screened using lower vertebrate animal models.

H. SUMMARY OF ANIMAL MODELS OF SPINAL FUSION

In summary of spinal fusion techniques and approaches, the posterior intertransverse process fusion (PLSF) is the most relevant and useful of the posterior fusion techniques for extrapolating data to the human condition. Facet and interlaminar fusions are achieved too easily in most lower animal models to be a useful test of spinal fusion implants. Challenging interventions such as the PLSF technique applied to lower animal models, may provide a useful and very inexpensive method to examine potential fusion enhancing biomaterials. The canine model has been the most widely used, is small enough to be cost effective and manageable, and is large enough to allow internal fixation and adequate iliac crest bone for harvest. For primarily biologic questions, however, the rabbit is cost effective and manageable, and still permits adequate iliac bone for harvest. The intermediate fusion rate associated with ICBG condition in this model is useful to the extent that it allows valid comparison of both positive and negative experimental conditions.

For anterior fusions, the sheep, goat, and dog have been used successfully. The larger disc spaces and vertebral bodies characterizing the sheep and caprine models are useful in the sense that sufficiently large implants or anterior fixation devices may be placed in the discectomy space.

The cervical spine models are essentially anterior cervical discectomy and fusion models and, in this regard, the alpine caprine model has been most widely used and validated. Most investigators using this model have preferred to use either an alternating two level fusion model or a contiguous three level fusion model, and a balance design to eliminate the influence of location.

For both anterior and posterior fusions, the most valid biologic model is the nonhuman primate, with its biology and the mechanics most closely simulating that of the human. This model, however,

is highly cost ineffective, challenging to manage, and, because of its evolved mental capacity, the most ethically difficult to use. Several nonhuman primate studies have demonstrated efficacy of rhBMP-2.^{90,91} Studies using these highly evolved mammals are conducted on a very selective basis and usually just prior to embarking on human clinical studies.

IV. CONCLUSIONS

The spine is one of the most frequently modeled parts of the skeletal system, in part because of its complexity, and in part because of its simplicity. The complex coupled motions of different segments of the spinal column, the assessment of spinal instability, and the effects of spinal fixation are not completely understood and continue to be rigorously investigated in a variety of selected animal models. On the other hand, because of the anatomic and kinematic consistency of selective spinal segments, interventions to the spinal column are often reliably predictable. In fact, creation of a spinal fusion is often a useful *preliminary* test of the biologic characteristics of an experimental implant meant for general skeletal repair.

As discussed in this chapter, numerous animal models have been employed to explore various aspects of spinal instability, spinal fixation, and osseous spinal fusion. Each animal model has its particular benefits and limitations. Still others are left to be explored. With the continued evolution of spinal fixation technology and our entrance into the era of biologic control of osteogenesis, the selection of appropriate and relevant animal models and surgical techniques for the study of adjuvant therapies in spinal fusion surgery becomes increasingly important.

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28 Animal Models of Spinal Cord Compression

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I. INTRODUCTION

Myelopathy due to chronic compression of the spinal cord is often encountered in cases of cervical spondylosis, ossification of the posterior longitudinal ligament (OPLL), cervical disc herniation, and spinal tumor, but the pathogenesis and pathophysiology of chronic compression myelopathy remain unclear or controversial.¹ The availability of experimental animal models that mimic the human diseases and consistently reproduce the diseases would materially facilitate basic investigations of the pathogenesis and pathophysiology. They could provide the basis for a more comprehensive analysis than is possible with humans.

Models of spinal cord compression were classified by Fehlings and Tator² as kinetic (acute) compression and static (chronic or subacute) compression according to biomechanics of the applied forces. Acute (kinetic) compression models involve rapid compression of the spinal cord in less than one second.² The applied force compresses the spinal cord with increasing velocity (with acceleration) to the point of maximal compression. Allen started modern research of acute spinal cord injury.³ He introduced a model of acute spinal cord injury which could be quantified and standardized. He dropped weights from various heights onto the surgically exposed spinal cords of dogs, and expressed the force of the contusion injuries in gram centimeters.³ This weight-dropping method became one of the most frequently used acute compression models, and numerous modifications have been developed. Fehlings and Tator² classified acute compression models as follows: weight-drop method, extradural balloon compression method, clip compression method, and vertebral dislocation method. A review of these models is described elsewhere.² Acute compression models attempt to simulate the biomechanical features of most types of acute spinal cord injuries, and do not seem to simulate myelopathy due to chronic compression of the spinal cord.

In contrast, chronic (static) compression models use forces which slowly compress and injure the spinal cord at an approximately constant velocity (without acceleration). Chronic compression models attempt to simulate mass lesions which gradually compress the spinal cord such as osteophytes, OPLL, herniated intervertebral discs, and spinal tumors. According to Fehlings and Tator,² two types of lesion-making methods can be considered in this category: the technique of graded addition of compression, and the use of slowly expanding mass lesions. In this chapter, these chronic compression models will be discussed.

II. SELECTION OF ANIMAL MODELS

Fehlings and Tator² called attention to the fact that many experiments of spinal cord injury from the literature contained serious errors in the experimental design. The main flaws were selection of an inappropriate model and/or species, inadequate sample size, and lack of objectivity.

The model of chronic spinal cord compression selected should be highly reproducible and appropriate for the particular animal species to be studied. Selection of an appropriate method for a particular species is sometimes difficult, and depends on intricacies of the methods and body size of the animal. For example, although advancement of a screw to produce spinal cord compression reported by Hukuda and Wilson⁴ may be reproducible and acceptable for experiments on larger animals such as dogs or primates, this method may be too intricate to be applied to smaller animals such as rats or mice.

Methods used to evaluate outcome of experimental spinal cord compression also should be considered when the model and species are selected. Clinical neurological evaluation such as Tarlov's scale⁵ or its modifications may be easier to perform on larger animals. Fehlings and Tator,² however, criticize the use of Tarlov's scale and its modifications in species other than primates because dogs and cats with complete transection of the spinal cord are capable of "spinal walking" and other forms of reflex limb movement, and these interfere with clinical neurologic evaluation of limb function. Functional clinical tests such as inclined plate,⁶ which seem to be consistent and quantifiable, may be more easily applied to smaller animals such as rats or mice. Histopathology of the injured spinal cord may be essential and can be applied to any of the models and species. Spinal cord blood flow (SCBF) can be measured in either large animals such as dogs and monkeys^{1,7} or smaller animals such as rats.⁸ Magnetic resonance imaging (MRI) may be capable of resolving the finer pathoanatomical features of the injured spinal cords of the larger animals.¹ Neurophysiologic tests such as measurements of somatosensory evoked potentials (SEP) or motor evoked potentials (MEP) may be very difficult and intricate on smaller animals.

III. COMMONLY USED MODELS

Selected reports on chronic compression models are listed in Table 1.

A. CLASSICAL MODELS

In the 1950s, Tarlov developed a method of gradual spinal cord compression in dogs by slowly inflating an extradural balloon.^{5,9-11} The balloon was introduced through a laminectomy defect at the level of T12, and threaded extradurally to the mid-thoracic region. The animals became paraparetic within 45 minutes and paraplegic within two hours. Doppman et al. described a percutaneous technique for producing intraspinal mass lesions in dogs and monkeys.⁷ Small balloon catheters introduced through needles into the spinal canal are positioned under fluoroscopic control to simulate epidural masses. Selective spinal cord angiography and silicone perfusion studies demonstrate the effect of such masses on SCBF. Croft et al. applied graded pressure by placement of weights directly

TABLE 1
Selected Reports on Chronic Spinal Cord Compression Models

1st Authors, Year ^{Ref.}	Animal Species	Method of Cord Compression
Tarlov 1953–1957 ^{5,9–11}	Dog	Balloon Inflation
Doppman 1973 ⁷	Dog, monkey	Balloon Inflation
Croft 1972 ¹²	Cat	Weights
Eidelberg 1976 ¹³	Ferret	Weights
Bennett 1977 ¹⁴	Cat	Casein Plastic Mass
Olsson 1958 ¹⁵	Dog	Disc Protrusion
Coman 1951 ¹⁷	Rat	Tumor Growth
Ushio 1977 ¹⁸	Rat	Tumor Growth
Aoki 1997 ¹⁹	Rat	Tumor Growth
Hukuda 1972 ⁴	Dog	Metal Screw
Hukuda 1988 ²⁰	Dog	Metal Screw
Schramm 1983 ²¹	Cat	Implantable Screw
Al-Mefty 1993 ¹	Dog	Teflon Screw and Washer
Miyamoto 1991 ²²	Mouse	Osteophyte and Disc
Miyamoto 1992 ³⁴	Mouse	Ossified Ligamentum Flavum
Saito 1992 ⁴⁰	Rabbit	Ossified Ligamentum Flavum

onto the spinal cord of cats exposed through laminectomy to produce reversible blocking of MEP and SEP as a means of estimating spinal cord damage.¹² Eidelberg et al. applied weights sequentially to the thoracic spinal cords of ferrets to produce a model of incomplete cord injury.¹³ Bennett and McCallum inserted a casein plastic mass into the epidural space at cervical spine levels of cats.¹⁴ The casein plastic mass slowly absorbed water and increased in weight and volume from 50 to 100% during the five to 18 days they were implanted, and produced spinal cord compression severe enough to result in loss of SEP.

B. HERNIATED INTERVERTEBRAL DISC

It is a well-known fact that certain chondrodystrophic breeds of dogs have a greater tendency towards degeneration and herniation of the intervertebral discs than other breeds.¹⁵ Olsson, a Swedish veterinarian, studied approximately 1300 clinical cases of disc herniations in dogs during an eight-year period, and found symptomatic cervical disc herniation in only 40 of these cases.¹⁵ This might mean that even in the chondrodystrophic breeds of dogs symptomatic cervical disc herniation is exceptional, and is not easily available as an experimental model.

C. TUMOR

According to Batson, the human vertebral venous system lacks valves and acts as a pathway of cancer metastasis.¹⁶ Based on this theory, Coman and DeLong injected a suspension of Walker 256 carcinoma cells into the tail veins of rats while applying abdominal pressure.¹⁷ Tumors grew in the vertebral venous system in twelve of fourteen rats, and six rats developed paraplegia.

To simulate extradural tumors in humans, Ushio et al. produced epidural spinal cord compression in rats by injection of Walker 256 carcinoma cell suspension anterior to the T12 or T13 vertebral body.¹⁸ Tumors grew through the intervertebral foramina to compress the spinal cord and produce paraplegia in three to four weeks. They could assess the effects of several treatments such as dexamethasone, radiation, laminectomy, and cyclophosphamide on clinical symptoms. This animal model appears to be useful for studying the treatment of human spinal cord compression produced by epidural neoplasms.

Aoki et al. placed 2-mm cubes of c-SST-2 mammary carcinoma in the epidural spaces of rats through a laminectomy defect.¹⁹ Forty-one of 45 rats developed paralysis of the hind legs. Paraparesis occurred 6–16 days after implantation of the tumor cells, and paraplegia occurred 6–21 days after implantation.

C. SCREW

Hukuda and Wilson performed a series of experiments to determine the pathogenesis of cervical spondylotic myelopathy.⁴ Their hypothesis was that vertebral osteophytes impinged on the spinal cord, and produced not only compression but also local ischemia of the spinal cord. This local vascular insufficiency was enhanced by disturbance in blood supply from systemic circulation to the spinal cord, and might then produce irreversible changes in the spinal cord, namely, myelopathy. They produced maximal tolerable compression of the spinal cord in dogs by advancing a screw through the anterior portion of C5 vertebral body into the spinal canal until limb weakness occurred. Chronic vascular insufficiency was established in the cervical spinal cord by blocking or ligating the anterior spinal artery, the vertebral arteries, and their branches in various combinations. From neurologic, microangiographic and, histopathologic findings, they concluded that the effects of vascular insufficiency and compression were synergistic.

Using a modified model of cervical spondylotic myelopathy, Hukuda et al. investigated the effects of several types of abnormalities such as systemic arterial hypotension, systemic arterial hypertension, cervical hyperflexion, cervical hyperextension, and cervical instability on histopathology of the spinal cord.²⁰ They produced the modified model of cervical spondylotic myelopathy using the following method. A dual screw was inserted into a drill hole piercing the C5 vertebral body of dogs, and under X ray control, an inner screw was gradually advanced into the spinal canal until it reduced the anteroposterior diameter of the canal by 45%. In addition, the vertebral arteries were obliterated bilaterally by inserting a catheter from C6 to the C2 level. They found pathologic changes of the spinal cord which were characteristic of each type of abnormality: peripheral necrosis of the central gray matter in systemic arterial hypotension, capillary congestion and subarachnoid hemorrhage in systemic arterial hypertension, and linear necrosis of the central gray matter and occluded anterior spinal artery in cervical hyperflexion. The pathologic severity was proportional to the number of applied abnormalities.

Schramm et al. applied implantable compression screws dorsally against L1 vertebral bodies of cats, advanced the screws by stepwise tightening at intervals of four to seven days, and used cortical and spinal evoked potentials to monitor the effect of the chronic cord compression.²¹ They found that neurological alternations appeared later than alterations in spinal evoked response but earlier than alterations in cortical evoked response.

Al-Mefty et al. achieved subclinical cervical cord compression in dogs by placing a Teflon screw anteriorly and a Teflon washer posteriorly, producing an average of 29% stenosis of the spinal canal.¹ They reported that twelve of fourteen dogs developed delayed and progressive signs of myelopathy, with a mean latent period to onset of myelopathy of seven months. It is noteworthy that this model of cervical spondylotic myelopathy allowed control of the spinal cord compression, an assessment of neurologic deficits, imaging evaluations like MRI, SEP recordings, SCBF measurements, and postmortem histopathologic examinations.

D. SPONDYLOSIS

The term “spondylosis” describes chronic degenerative lesions of multiple or single intervertebral discs and the consequent osteophytosis of related vertebral bodies. Spondylosis is an important cause of musculoskeletal disability in humans: the disc degeneration leads to the clinical syndromes of radiculopathy and myelopathy. Miyamoto et al. established an experimental model

of cervical spondylosis in rodents with the working hypothesis that mechanical instability in the spine would induce disc degeneration and spondylosis.²²

Fifty-seven adult (six-month-old) male ICR strain mice weighing approximately sixty grams were used. They were divided into two groups: thirty mice for the experimental group and twenty-seven mice for the control group. Each group was further divided into three subgroups as described below. Thirty mice in the experimental group were surgically treated with the following procedures. The back paravertebral muscles of the mice were detached from the spinous processes, laminae and facets of the cervical, thoracic and lumbar vertebrae. Next, the spinous processes together with supraspinous and interspinous ligaments of the cervical, thoracic and lumbar vertebrae were all resected. The skin was closed without reattachment of the paravertebral muscles so that these muscles could not work as they used to. After recovery, the animals were allowed to move freely about their cages. The 27 mice in the control group were not treated at all in order to observe the aging process of the spine.

Ten of the thirty mice in the experimental group were sacrificed two months after operation (E2M group) to observe short-term effects of the surgical intervention, and nine of the twenty-seven mice in the control group were sacrificed and examined at the same time (C2M group). Another ten mice in the experimental group were sacrificed six months after operation (E6M group) to observe mid-term effects of the surgical intervention while another nine mice in the control group were sacrificed simultaneously (C6M group). The remaining ten mice in the experimental group were sacrificed twelve months after operation (E12M group) to observe long-term effects while the remaining nine mice in the control group were also sacrificed (C12M group). All the animals were killed by carbon dioxide or ether inhalation.

Radiographic studies were performed to examine disc-space narrowing, osteophyte formation and changes in the spinal alignment. The entire vertebral column was quickly dissected free and the cervical and upper thoracic spine was examined radiographically along the anterior-posterior and lateral planes. The radiographic examinations showed that in the C2M, C6M and C12M groups no disc-space narrowing, osteophyte formation or abnormal spinal alignment was observed. No evidence of osteophyte formation was seen in the E2M and E6M groups. In all the cervical vertebral columns in the E12M group, however, lesions were characterized by disc-space narrowing and/or anterior osteophyte formation most prominently seen at C4-5, C5-6 and C6-7 levels. Pathologic cervical kyphosis was also observed in half of the specimens in the E6M and E12M group.

Histologic studies were performed to examine the degeneration process of the intervertebral discs, osteophyte formation, and spinal cord compression. The entire dissected vertebral column of each specimen was fixed in neutral buffered formalin, decalcified in formic acid, split mid-sagittally, embedded in paraffin, sectioned, and stained for the light microscopic examination. Histologic examinations revealed that most of the intervertebral discs appeared normal in the C2M and C6M groups. In contrast, most of the intervertebral discs in the C12M and E2M groups exhibited proliferation of cartilaginous tissue and loss of lamellar structure in the anterior annulus fibrosus. Shrinkage or disappearance of the nucleus pulposus and clefts or fissures in the annulus fibrosus were observed in several discs. In the E6M group, disc degeneration was more advanced and the lesions were characterized by shrinkage or disappearance of the nucleus pulposus and fissures in the annulus fibrosus. Herniation of disc materials and anterior osteophyte formation were observed in several discs. In the E12M group, disc degeneration was most advanced and the lesions were characterized by herniation of disc materials and anterior osteophyte formation. Posterior herniation of disc materials led to impingement on and deformity of the contiguous spinal cord (Figure 1).²²

Although the degree of spinal cord compression was not severe and neurological deficits were evident in none of the animals, the authors believe that this experimental model could be valuable in helping to understand the pathoanatomy and pathophysiology of myelopathy due to static compression of the spinal cord caused by cervical spondylosis or disc herniation.



FIGURE 1. A mid-sagittal section shows that herniation of disc tissue was compressed and deformed in this model (Masson stain). Neurons in the gray matter at the compressed level seem to be reduced in number.

E. VERTEBRAL HYPEROSTOSIS

Both ankylosing spinal hyperostosis (ASH)²³ and diffuse idiopathic skeletal hyperostosis (DISH)²⁴ are well-known conditions in which hyperostosis is associated with ossification of the ligaments of the spine. Patients with ASH or DISH often develop ossification of the posterior longitudinal ligament (OPLL)^{25,26} and/or ossification of ligamentum flavum (OLF)^{27,28} causing serious neurological complications. Protrusion of the thickened, hypertrophied and ossified ligaments into the spinal

canal leads to compression and deformation of the contiguous spinal cord and nerve roots, which cause myelopathy and radiculopathy. Histopathologic studies of OPLL²⁵ and OLF²⁸ have revealed that the development and growth of OPLL and OLF are based on hypertrophy of the ligamentous tissues with proliferation of fibrocartilaginous cells. The regular fibrous matrices and frame work are disrupted in OPLL and OLF, and collagen fibers increase in number and size.^{25,28} Numerous fibrocartilaginous cells are found in the increased collagenous matrices. Some parts of the ossified mass are almost always in continuity with the posterior cortex of the vertebral body in OPLL, and with the lamina in OLF. The ossification extends mainly along the superficial layer of the hypertrophied posterior longitudinal ligament and ligamentum flavum. There are many cartilaginous cells with abundant matrices and nutritional vessels at the area of the ossification front. These histologic findings may indicate that OPLL and OLF have developed through a process of endochondral ossification. In some parts, bone apposition toward the spinal canal is found on the surface of the ossified ligaments.²⁵ There is no evidence of inflammation in the hypertrophied or ossified ligaments. In the pathogenesis of ossification at the juxta-skeletal sites such as OPLL and OLF, growth factors which can initiate and stimulate new cartilage and bone formation may be important.²⁹

During the past decade, studies have shown that a number of growth factors regulate the development, growth and maintenance of cartilage and bone tissues.^{29,30} Among them, bone morphogenetic proteins (BMPs) and transforming growth factor- β s (TGF- β s) may have more important roles on the pathogenesis of OPLL and OLF, because BMPs initiate cartilage and bone differentiation and induce new cartilage and bone formation *in vivo*, whereas TGF- β s stimulate cartilage and bone formation by determined chondroprogenitor and osteoprogenitor cells *in vivo*.³¹ BMPs were originally defined as bone-inducing substances responsible for new cartilage and bone formation at the extra-skeletal sites by intramuscular or subcutaneous implantation of demineralized bone matrix.^{29,32} The process of new cartilage and bone formation induced by the implantation of BMPs is a sequential cascade of several events: activation and migration of undifferentiated mesenchymal stem cells; attachment of the cells to collagenous matrices; proliferation of the mesenchymal stem cells; differentiation of the stem cells into chondrocytes; maturation and hypertrophy of the chondrocytes and mineralization of the cartilage matrix; angiogenesis and vascular invasion; new bone formation via endochondral ossification; remodeling of the bone and hematopoietic marrow formation in the ossicle.³³

Because some BMPs have bone-inducing potentials as described above, they may be causative factors in the development and growth of OPLL and OLF. This idea led Miyamoto and coworkers to determine whether BMPs could experimentally induce OLF and secondary spinal cord compression in animals.³⁴

Forty-eight adult (24-wks-old) male ICR strain mice were used for the experiment. These mice were divided into two groups; 30 mice in the experimental group and 18 in the control group. Partially-purified BMP fraction was prepared from a murine osteosarcoma (Dunn) as previously described.³⁵ This BMP-active fraction was confirmed to contain murine BMP-4.^{35,36} The partially-purified murine BMP-4 was mixed with telopeptide-depleted bovine skin type I collagen in a 0.01 M hydrochloride solution and then lyophilized. The collagen was used as a delivery system for murine BMP-4 which prevented rapid outward diffusion and permitted sustained release.³⁷

Thirty mice in the experimental group were surgically treated with the following method. A longitudinal skin incision was made over the lumbar vertebral column, and the dorsal paravertebral muscles were detached from the spinous processes and laminae. The supraspinous and interspinous ligaments of L2-3 or L3-4 were resected. A mid-sagittal slit was made between the right and left ligamenta flava. Using a surgical microscope, two tiny and thin sheets of the BMP-4/collagen composite (100 mg) were inserted into the posterolateral epidural space through the slit, and thereby, in the epidural space, one of the BMP-4/collagen sheets was on the surface of the right ligamentum flavum and the other the left. Eighteen mice in the control group were surgically treated with the same procedure except that collagen (100 mg) without the murine BMP-4 fraction was inserted into the same location in the epidural space.

The 30 mice in the experimental group were sacrificed four, six and eight weeks (10 mice for each time period) after implantation of the BMP-4/collagen composites. They were named E4, E6 and E8 groups, respectively. The 18 mice in the control group were also sacrificed at the same intervals (six mice for each time period). Blocks of whole lumbar spinal columns containing the implanted site were resected together with the paravertebral muscles. They were radiographically examined, and then the specimens were fixed, decalcified, cut sagittally or transversely, embedded, and sectioned. Serial sections were stained with hematoxylin and eosin, toluidine blue, van Gieson's solution, and Kluver-Barrera's solution for light microscopic examination.

Radiographic examination of the spinal columns showed that, in all the mice of the experimental group, a pair of beak-like calcified prominences arose from the laminae at the BMP-4/collagen-implanted segment and protruded into the spinal canal on a lateral radiograph.³⁴ The cephalic and caudal parts of these bony prominences did not unite completely, even the largest. The degree of narrowing of the spinal canal caused by the bony prominences was obtained by dividing the width of the bony prominences by the diameter of the spinal canal measured on a lateral radiograph. By this calculation, the degree of narrowing was $34.7 \pm 10.9\%$ (mean \pm SD) in the E4 group, $42.8 \pm 12.3\%$ in E6, and $46.4 \pm 13.9\%$ in E8. Between the E4 and E8 groups, there was a significant difference in the degree of narrowing of the spinal canal caused by the bony prominences ($p < 0.05$, Student's *t* test). This indicated that the size of the bony prominences had increased gradually with the lapse of time. In the control group, there was no abnormal shadow on radiographs.

Histologic examinations of the ligamenta flava showed that, in all the mice of the E4 group, the ligamentum flavum became hypertrophied and a pair of newly formed bony prominences protruded into the spinal canal from the ventral (canal-side) surfaces of the contiguous laminae. The bony prominences adhered to the dura mater and led to compression of the contiguous spinal cord. Fibrous or cartilaginous tissue was found to intervene between the cephalic and caudal parts of the ossified loci. The ossification appeared to extend along the ventral (superficial) layer of the hypertrophied ligament because the ossification was more advanced in that layer. The ventral (superficial) layer was gradually replaced by newly formed bone and cartilage tissues, whereas in the dorsal (deep) layer, collagenous fibers were irregularly hyalinized and elastic fibers were partially disappeared and disrupted. In all the mice in the E6 and E8 groups, endochondral ossification was more advanced. The ossified areas extended further along the hypertrophied ligament but their cephalic and caudal parts did not unite completely with the intervening fibrous and cartilaginous tissue. A small amount of original ligamentous tissue, in which elastic fibers were decreased in number and scattered within newly formed fibrocartilaginous tissue, remained in the dorsal (deep) layer. In the control group, the fibrous constructs of the ligament appeared intact and no new bone or cartilage formation was observed.

Histologic examinations of the spinal cords showed that, in the E4 group, the spinal cord exhibited deformation secondary to compression by the protruded ossified ligamentum flavum (See Figure 5 in Ref. 34). In spite of this mild deformation, little or no degenerative change was evident in the spinal cord. In the E6 and E8 groups, the spinal cord exhibited marked deformation. In those cases with moderate deformation of the spinal cord, demyelination was evident in the posterior and lateral white columns while in the anterior white columns it was not.³⁴ The gray matter appeared intact; neuronal loss or chromatolysis in the anterior horn was not observed.³⁴ In those cases with severe deformation of the spinal cord, both the white and the gray matter degenerated (Figure 2).³⁴ Demyelination and loss of axonal fibers in the posterior and lateral white columns were marked, while little demyelination was observed in the anterior white column (Figure 2).³⁴ Neuronal loss and chromatolysis in the anterior horn were also evident (Figure 2).³⁴ These degenerative changes in the spinal cord were, on the whole, more severe in the E8 than in the E4 group. In the control group, no spinal cord was deformed or degenerated.

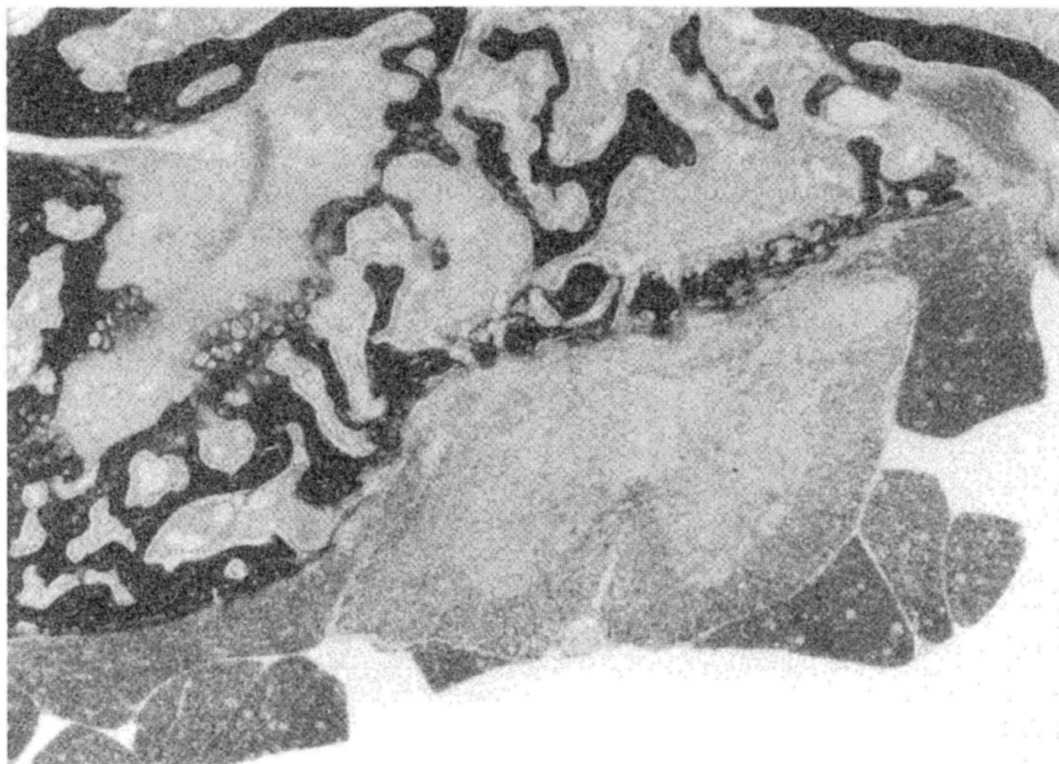


FIGURE 2. A transverse section shows severe deformation of the spinal cord by ossified ligamentum flavum (toluidine blue stain). Demyelination and loss of axonal fibers are predominant in the posterior and lateral columns.

The authors concluded that OLF could be experimentally induced in mice by the bone-inducing effect of murine BMP-4. This experimental study has provided direct evidence that the stem cells which can respond to the cartilage-inducing and bone-inducing signals of murine BMP-4 and, consequently, differentiate into chondrocytes and osteoblasts do exist in or around the ligamenta flava of mice. Another important outcome is that the pathologic findings in this model bear close resemblances to those reported in clinicopathologic studies of OLF.^{28,38,39} In both the present experimental study and the clinicopathologic studies, the following have been found: ossification is accompanied by degeneration and hypertrophy of the ligament; the bony prominences arise from the ventral (superficial) surface of the laminae and extend along the ventral (superficial) layer of the hypertrophied ligament; the cephalic and caudal parts of the ossified areas do not unite completely with the intervening fibrous or cartilaginous tissue; the ossified ligament develops through a process of endochondral ossification; the ossified ligamentum flavum increases in size over time and causes gradual compression and deformation of the spinal cord leading to the pathologic changes. These results may indicate that BMPs play an important role in the development and growth of not only OLF but also OPLL. However, because the murine BMP-4 fraction used in this experimental study was not completely pure, it might have contained minor contaminants such as TGF- β s. It remains a possibility that TGF- β s may also play a role in the development and growth of the ossification.

In addition, this experimental model could be useful for the pathoanatomical and pathophysiological study of myelopathy due to gradual chronic compression of the spinal cord caused by OPLL or OLF. Saito et al. used rabbits and a crude fraction of unidentified BMP-like substances to simulate OLF, and showed similar outcomes.⁴⁰

IV. CONCLUDING REMARKS

The availability of an experimental animal model that mimics the human disease and consistently reproduces the disease would facilitate basic investigations of pathogenesis and pathophysiology, and moreover, would be useful to test or compare effectiveness of various treating modalities. It could provide the basis for a more comprehensive analysis than is possible with humans. Although many models of spinal cord compression have been reported so far, there have been few reports on the test or comparison of various treatment modalities using those models.¹⁷ Development of more reproducible and less complicated models might be required in order to test or compare the surgical or non-surgical treatment modalities.

Although cervical spondylosis, OPLL, cervical disc herniation, spinal tumor, and spinal cord tumor may be all similar in terms of spinal cord compression, each has its own characteristic features. For example, hard osteophytes produce multi-segmental cord compression in most cases of cervical spondylosis whereas soft discs cause single-level compression in many cases of cervical disc herniation. Does a model of spinal cord compression by a single metal or plastic screw simulate cervical spondylosis or disc herniation? It may be necessary to develop a specific model which has characteristic features of a specific human disease causing spinal cord compression.

Evaluation of outcome is an extremely important aspect of models of spinal cord compression. Methods for the evaluation include clinical, anatomical, physiological, pathological, and radiological techniques. These outcome parameters should be objective, consistent, and quantifiable. Great care should be taken in the experimental design and the analysis of data.

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29 Animal Models for Reconstruction of Vertebral Column and Intervertebral Disc

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I. INTRODUCTION

The development of spinal surgery has offered a variety of solutions for the treatment of various spinal diseases or conditions. For segmental spinal defects (due to tumors or other pathological conditions), replacement of vertebral body with autogenic bone, allogenic bone,¹ bone cement, metal, or other biomaterials such as hydroxyapatite or ceramics^{2,3} has been reported (see Chapter 27 for spinal fusion methods). However, complications are found associated with spine fusion such as the degeneration of adjacent segments due to overloading. Therefore, the necessity of spinal reconstruction with physiological mobility having intervertebral disc function (dynamic spinal reconstruction) has been realized and several animal experiments have been conducted to evaluate the efficacy of different biological grafts or prostheses.

In order to restore the mobility of a natural spinal column with functional intervertebral disc, allografts, autografts, and artificial intervertebral disc prostheses have been investigated in animal models. The ultimate purpose is to search for reliable spinal prostheses leading to future clinical application. Clinically, several disc prostheses have been used for human patients with predominantly degenerative disc disease. Several prostheses have been reported, such as the Charite SB disc.⁴

It has been noticed that animals useful for experiments are those of bipedalism because their weight is loaded on the spine, while experimentation using quadrupedal animals cannot provide sufficient data. However, at present, it is extremely difficult to conduct an animal experiment using bipedal animals (nonhuman primates), so most researchers are using quadrupedal animals, such as dogs or sheep. In this chapter, spinal reconstruction without mobility and dynamic spinal reconstruction having intervertebral disc function will be described.

TABLE 1
Animal Models of Vertebral Body and Intervertebral Disc Replacement

Procedure	1st Author, Year ^{Ref.}	Animal	Prosthesis	Level	Time Period
Vertebral body	Waku 1990 ⁵	Dogs	Ti plate + cement	L4	16 wks.
Allogenic disc unit	Olson 1991 ⁶	Dogs	Stored allograft	T7-9	18 mo.
	Katsuura 1994 ⁷	Dogs	Stored allograft	Middle L	48 wks.
	Matsuzaki 1996 ⁸	Dogs	Stored allograft	L4-6	3 yrs.
	Frick 1994 ⁹	Dogs	Fresh autograft	L2/3,L4/5	4 mo.
Autogenic disc unit	Luk 1997 ¹⁰	Monkeys	Fresh autograft	L3/4,L4/5	12 mo.
	Hou 1991 ¹¹	Monkeys	Artificial disc	L4/5	15 mo.
Disc prosthesis	Vuono-Hawkins 1994 ¹²	Dogs	Artificial disc	L2/3,L5/6	12 mo.
	Nakamura 1997 ¹³	Dogs	Artificial disc	L3/4,L5/6	3 mo.
	Kostuik 1997 ¹⁴	Sheep	Artificial disc	Lumbar	6 mo.

II. ANIMAL MODELS AND ANIMAL SELECTIONS

Large animals such as dogs, sheep, and monkeys, have been used for experimentation of vertebral column and intervertebral disc graft or prosthesis (Table 1). In an immobilized spinal reconstruction, only the replacement of a vertebral body is done, so quadrupedal animals such as dogs or sheep may be appropriate. The evaluation of the function of intervertebral disc is not applied in this case, and only the healing of the graft or prosthesis to the adjacent vertebral body is concerned. Goats and pigs may serve as experimental animals for reconstruction or replacement of spinal columns. Because instrumentation (internal fixations such as plate, screw, etc.) is needed for spinal reconstructions, small animals, such as mice, rats and rabbits, are inappropriate simply due to their small bone volume.

In dynamic spinal reconstruction, the long term results of intervertebral disc function under similar weight-bearing conditions to humans is essential. Therefore, bipedal animals, such as monkeys, should be selected. However, due to their cost and limited availability, quadrupedal animals have been used. Although chickens are bipedal, they are too small to qualify.

III. SPINAL COLUMN RECONSTRUCTION WITHOUT INTERVERTEBRAL DISC

Using seven adult mongrels, Waku replaced the vertebral body alone using a special plate prosthesis made of a titanium alloy.⁵ The prosthesis was used to sustain the vertebral body with processes near the upper and lower ends. By intruding forward, L4 and its upper and lower intervertebral discs are excised, preparing furrows at the front of the L3 and L5, the prosthetic plate was put into it and filled with bone cement. Animals were killed four and 16 weeks postoperatively, L3 and L5 were taken out as en bloc and radiographically and histologically examined.

Although dislodgment of artificial vertebral body was not observed, submerging of artificial vertebral body into fixed vertebral body was found in all cases. However, when periodically examined, at the axial loading point of the end of vertebral body, the foregoing endplate was absorbed and by a large amount of new bone formation, an endplate thicker than normal was formed. The newly formed bone remained premature at four weeks and became a layer of trabecular structure at 16 weeks. The results indicated that by an appropriate loading, the remodeling of bone became marked.⁵

IV. DYNAMIC SPINAL RECONSTRUCTION

For restoring normal spinal function, allogenic and autogenic grafts of intervertebral disc with the adjacent vertebral bodies (a part of the vertebral body, about 1.0–1.5 cm thick on each side of the disc to be grafted), namely vertebral body/intervertebral disc complex, have been reported.^{7,8} An alternative to allografts is the fast development of artificial intervertebral disc prostheses. Major animal models for the above-mentioned applications are described as the following.

A. ALLOGRAFT

1. Multi-Segment Graft

Using 15 large adult mongrels (20–25kg), Olson et al.⁶ freeze stored large spinal graft units from T7 to T9 at 80°C for two weeks, excised the 8th rib and reached the vertebral body by transthoracic approach, cutting the vertebral bodies from T7 to T9 into a triangular form, and grafting the unit so it would not dislodge forward. In this procedure, no instrument was used to stabilize the unit. After 18 months, there was no biomechanical difference from normal cases. However, bone strength of the grafted area decreased significantly as compared with that of the normal area ($p < 0.05$). The bone union was obtained histologically, but at the middle vertebral body (T8), an incomplete revascularization was noted and the stratified structure of the intervertebral disc disappeared, showing progress of its denaturation.⁶

2. Katsuura's Method

In 13 adult mongrels, Katsuura et al.⁷ used allogenic grafting units attaching parts of the vertebral body to lumbar intervertebral discs which were stored at –80°C for four weeks in a programmed freezing system. Intruding transperitoneal abdominally, and after excising parts of the intervertebral disc and vertebral body, the unit was grafted. The unit was fixed by fixing the partial vertebral body plates to the upper and lower vertebral bodies. During three months until the evulsion of the plate was made, there was no mobility of the grafted intervertebral disc, so that there was a risk of marked denaturation of the disc. The metabolic activity of the intervertebral disc after four weeks of frozen storage decreased up to 44% of the fresh cases in terms of ³⁵S-sulfate incorporation. This means that because of freezing, the function of the intervertebral disc cell markedly decreased. Further, radiographically, narrowing of the intervertebral disc cavity progressed. These were closely similar to our results.¹

3. Matsuzaki's Method

The authors reported a basic procedure of allogenic intervertebral disc grafting in dogs.⁸ The lumbar spine (L1–7) was removed using a sterile technique from 11 mongrel dogs (15 kg average BW) to prepare disc units for transplantation. In order to fix a disc in the recipient lumbar spine without fail, it was removed with adjacent vertebrae transected about 1 cm apart from the surfaces facing the disc so that the cut surfaces could be fixed to the cut surfaces of the recipient vertebrae by bone fusion (Figure 1). Eight disc units were immersed for impregnation in 10% dimethyl sulfoxide (DMSO) at 4°C for one hr, and stored at –80°C for 1–16 weeks (mean, eight weeks). Another group of eight disc units were immersed for impregnation in 10% DMSO at 4°C for three hours, and frozen at –30 for one hr and further at –80°C for one hr, and then stored in liquid nitrogen at –196°C for 1–6 weeks (mean, four weeks) until transplantation.

Recipient animals were anesthetized intravenously with Nembutal, and the lumbar vertebrae were exposed through a peritoneal approach. The frozen disc unit was promptly thawed at 37°C in a thermostatic chamber and immediately transplanted. The disc unit was inserted mainly in the L4/5 or L5/6 intervertebral space. The disc to be replaced was removed with the adjacent vertebral

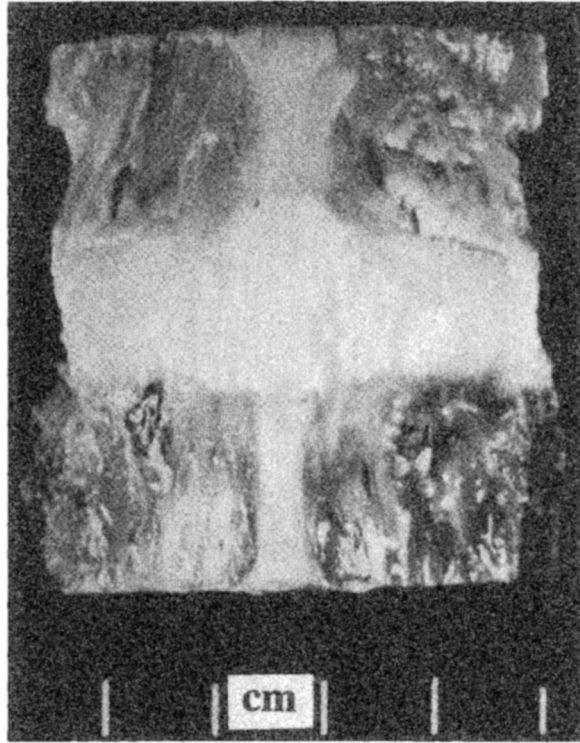


FIGURE 1. A donor disc unit removed with the adjacent vertebrae transected about 1 cm apart from the surfaces facing the disc.

bodies transected at levels about 1 cm apart from their surface facing the disc to be removed. The recipient bed was prepared carefully particularly so as not to injure the dura. The remaining vertebral bodies were cut so that the space between them could match the disc unit to be inserted. An AO mini plate was fixed to the inserted disc with two screws, leaving an about 1-cm long segment of the plate above the transected surface of the graft vertebral body so that it could be fixed to the recipient vertebral body. The recipient bed was trimmed, and the plate was fixed to the recipient vertebral body with screws (Figure 2).

At six months, bone fusion was accomplished in all animals according to radiographic evaluation. The fusion did not vary depending on the length of storage of the units. The disc units stored at -196°C showed less bone resorption than those stored at -80°C , and we had an impression that they achieved bone fusion somewhat faster. The disc space was not changed at six months, and its apparent narrowing appeared at around 12 months in both groups. The narrowing progressed gradually. At three and five years, the space became remarkably narrow with growth of osteophytes, indicating that the disc unit lost the normal disc function, although the disc space remained. Radiographs obtained with soft X rays also showed an adequate intervertebral space.

MRIs were obtained with lumbar spines removed from the recipients at six and 36 months. With specimens removed at six months, high signal areas representing the transplanted discs were demonstrated by T2 weighted, but the recipient discs and fresh discs also had the similar signal intensity to one seen in the transplanted disc. In the vertebral components of the unit, areas of high and low signal intensities were mixed (Figure 3). In other words, bone was not adequately mature in the transplanted unit. At 36 months, as at six months, the transplanted disc had the same high signal intensity as the adjacent discs. The bony portions of the transplanted vertebral component showed no change in signal intensity as observed at six months, indicating that the transplanted bone had been completely replaced with newly formed bone.

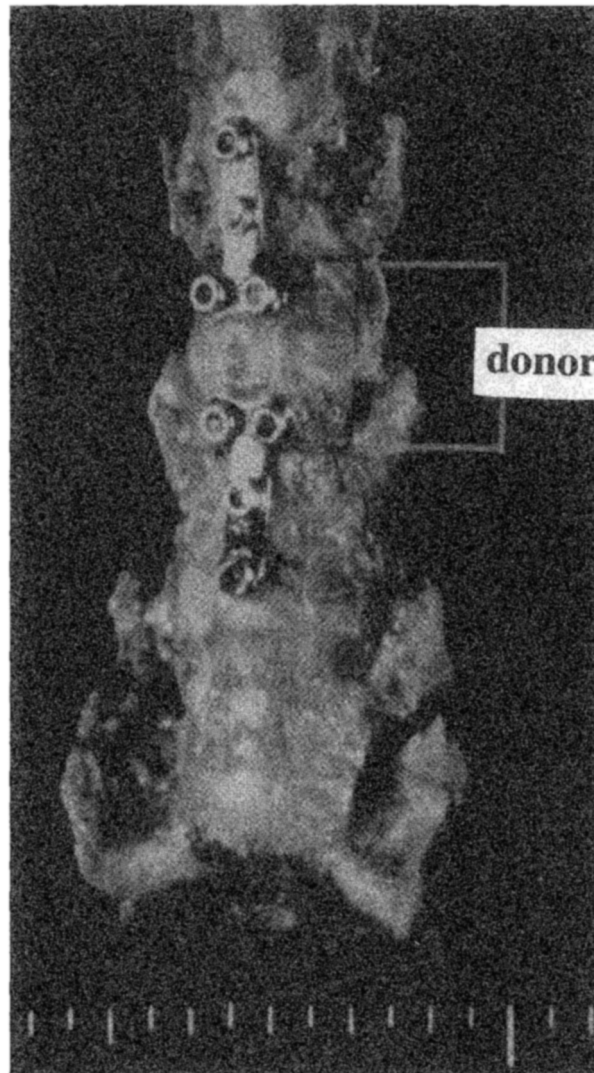


FIGURE 2. The fixation of the disc unit was made with two plates and screws. The disc in the donor unit (between the two lines) remains mobile.

Before transplantation, for the disc units stored at both temperatures, vacuolation and other features of degeneration were observed in the nucleus pulposus using histological examination. However, the annulus fibrosus was morphologically well preserved, particularly for the units stored at -196°C . The changes were not dependent on storage times in both groups.

Discs removed from recipients at six months were decalcified, stained with HE, and examined. With disc units stored at -80 and -196°C , the graft-recipient bone interface showed excellent bone fusion. Cells decreased markedly in number, however, with fibrosis in bone marrow of the disc unit. No lymphocyte was observed, nor was there any evidence suggestive of rejection.¹⁵ Regardless of store temperatures, the normal structure of the nucleus pulposus was maintained in the disc unit, but cells without the nucleus were distributed evenly, practically without infiltration of lymphocytes. The disappearance of the nucleus was remarkable in the annulus fibrosus, which was slightly edematous, but had the normal lamelliform structure. Storage at -196°C is more suitable for the preservation of the annulus fibrosus than that at -80°C .

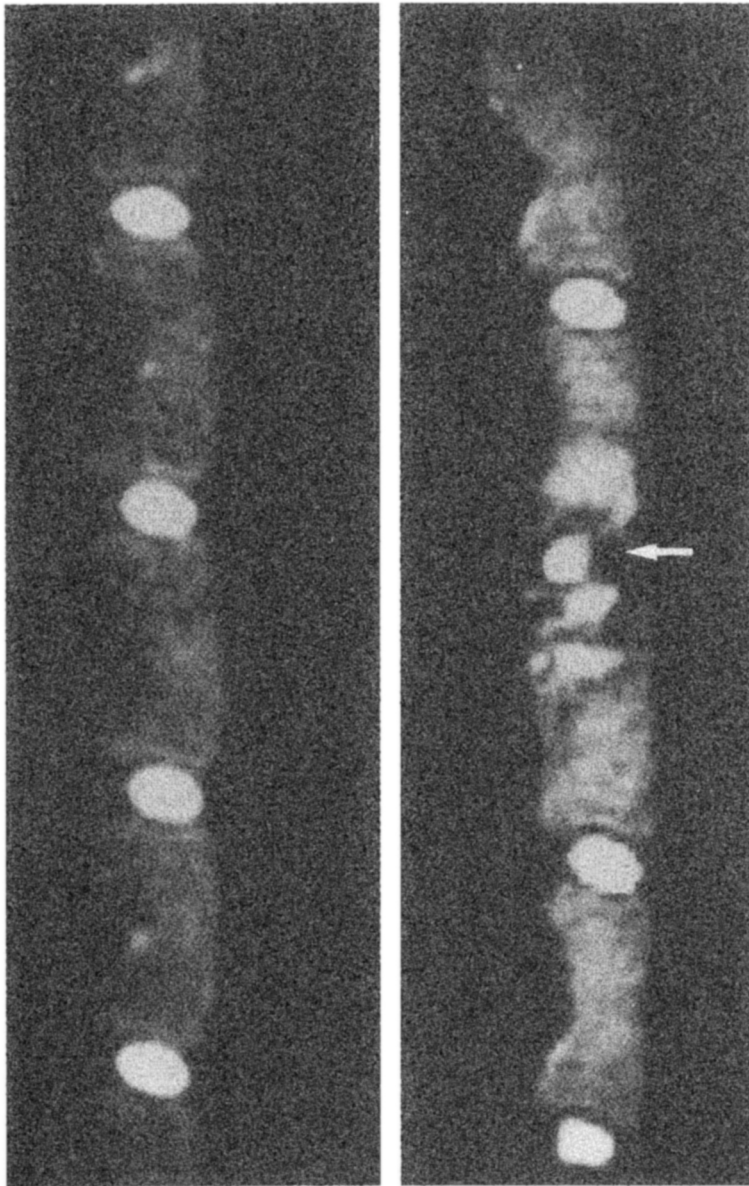


FIGURE 3. MRI images of a fresh disc (A) and at six months after the transplantation (B). The high signal area represents the transplanted disc (arrow).

The cell activity in the transplanted intervertebral disc was also assessed at three and six months. In disc units stored at -80 and -196°C , both incorporation of ^{35}S -sulfate and ^3H into cells and their liberation from the cells into the supernate were markedly decreased. When assessed by incubation of the cells for 12 hours, the cell activity of the transplanted discs was $1/5$ to $1/20$ of that of fresh intervertebral discs. With an expectation that their incorporation and liberation would increase if cells were active, the cells were cultured further for 24 hours, but neither ^{35}S -sulfate or ^3H -proline was incorporated in, and liberated from, the cells any more, suggesting that the cells were not active or dead.

B. AUTOGRAFT

Similar procedure was employed in the several investigations of autogenic transplantation of intervertebral disc units. Because of their autogenic nature, there was no immunological reaction, therefore, pure changes in intervertebral disc grafting can be evaluated.

Using eight adult mongrel dogs (23–30kg), Frick et al.⁹ performed an autogenic intervertebral disc transfer. Grafting was done by exchanging L2/3 and L4/5 discs. The discs were excised and thin sections (2 to 4 mm in depth) were prepared for grafting. The grafted intervertebral discs were fixed with wire so that mobility of the disc was preserved. Animals were killed after four months. Their spines were longitudinally incised, and mobility, histological change, and biochemical parameters were examined. The synthesis (³⁵S) of proteoglycan was preserved in the annulus fibrosus, but decreased in the nucleus pulposus ($p < 0.05$). However, no change in DNA content was observed. Histologically, revascularization and remodeling were observed in the donor bone. The structure of the annulus fibrosus was preserved but the endplate changed irregularly.⁹ From these facts, it was suggested that the intervertebral disc once cut off does not completely recover to normal, and the trend is high in the nucleus pulposus. The reason that the result was somewhat unfavorable in comparison with that of Luk et al.¹⁰ may be possibly due to differences in experimental animals and grafted intervertebral disc units. However, these tests are said to be of value for discussing the fate of grafted sections.

Luk et al.¹⁰ performed an autografting of intervertebral disc in rhesus monkey. The rhesus monkey is a bipedal animal, so the results are interesting. To the upper and lower parts of a lumbar intervertebral disc, a thin section vertebral body of 1.5 mm in depth was attached and excision of intervertebral disc was conducted, thereafter, replaced at the same site followed by 12-month observation. The bone union was obtained within two to four months, and though narrowing of the intervertebral disc space was temporarily observed, it recovered normally after 12 months. Histologically, only a mild denaturation was observed and the decrease in proteoglycan of the nucleus pulposus in the early period was gradually re-accumulated. The large mobility of the grafted intervertebral disc in the early stage of grafting gradually stabilized. From these facts, it was suggested that cut-off intervertebral disc decreases its function in the early stage because of ischemia but thereafter recovers. However, autograft of the intervertebral disc is inappropriate for clinical application.

C. ARTIFICIAL INTERVERTEBRAL DISCS

Compared to allogenic and autogenic grafts, intervertebral disc prostheses have shown their bright future because of their availability. Over the last several decades, a tremendous effort has been made to develop an artificial disc to replace a degenerated disc. The goal is the restoration of the natural mechanics of the segment after disc excision, thus relieving pain and preventing further degeneration of adjacent segments.

Hou et al.¹¹ placed artificial intervertebral discs made of silicon rubber in lumbar spines of four adult monkeys. After operation, intervertebral disc space was preserved. Histologically, the periphery of the artificial intervertebral disc was covered with fibrous tissue. It is highly possible that, after a long period, problems such as breakdown of silicone rubber may occur.

Vuono-Hawkins et al.¹² prepared 3-layer elastomeric intervertebral disc spacers for grafting into 12 dogs (20–30kg). The surface layers of the spacers were composed of hydroxyapatite. The plan was to replace the inner layers of the annulus fibrosus and nucleus pulposus, not completely replace the intervertebral disc. Under mechanical testing, compressive and torsional stiffness decreased to 25–42% of levels seen in control animals. The bone ingrowth was unfavorable on the surface of the hydroxyapatite. Much connective tissue was noted. Early migration was observed in 5 of 12 dogs.

In an experiment by Nakamura et al.¹³ an artificial intervertebral disc was developed and used for lumbar fixation in beagles. The intervertebral disc spaces were kept at favorable conditions at least one year. The artificial disc was composed of annulus fibrosus, nucleus pulposus and hydrous polyvinyl alcohol hydrogel (PVA-H). The area that affixed to the bed was a titanium fiber mesh with 70% porosity. The mesh was impregnated with PVA-H and gelatinized by low temperature crystallization. Infiltration of neogenetic bone was observed three months after the operation. Results of compression and torsion testing indicated a 2/3 decrease compared to a normal intervertebral disc. PVA-H is the most likely material for artificial intervertebral discs. By adjusting water content, it is possible to develop an artificial disc similar to a normal one. It is of great significance that the device can attach tightly to the vertebral body bed with titanium mesh.

Kostuik et al.¹⁴ inserted artificial intervertebral discs composed of metal and springs into lumbar spines of six ewes. Six months after the replacement, the device combined with the bed of the vertebral body and mobility of the disc was observed. The device was durable mechanically, but use of the spring carries the risk of debris accumulation.

IV. DISCUSSION AND CONCLUSION

Spinal reconstructions are important procedures for many pathological conditions. For the vertebral body, autografts, allografts and biomaterials can be used for clinical reconstruction of spinal column defects due to the removal of tumors. For intervertebral disc replacement, autografts, allografts and artificial intervertebral discs have been studied. It has been realized that a better method for spinal reconstruction is replacement of the intervertebral disc, aiming for dynamic spinal function. This is a relatively new area with only a limited number of experimental reports, so many questions remain unanswered.

Autografting an intervertebral disc has no clinical application because there are no donor sites in the human body. Therefore, the alternatives are restricted to allograft or artificial intervertebral disc. It is difficult to preserve the intervertebral disc function of allograft for a long period because of the decrease in cell function (including cell death) due to freezing and progress of degeneration after grafting. In addition, immunorejection may play an important role in the degeneration process. Therefore, intervertebral disc allografts are only useful as spacers for a limited period of time and are unlikely to be functional motion segments.^{8,9}

For artificial disc replacement, different materials have been used including polyethylene and metal,^{16,17} titanium (Kostuik prosthesis),¹⁴ polyurethane elastomer reinforced with Dacron fibers,¹⁸ silicone rubber,¹⁹ a hyaluronan-containing device,²⁰ and a hydrogel.²¹ Although a certain number of clinical reports have appeared, there are many recognized and potential problems and questions, for example, durability of materials,¹⁴ wear debris,^{14,22} toxicity (Link prosthesis),⁴ stability of the fixation (migration or dislocation),²³ or collapse of the prosthesis into the vertebral body.¹⁶ More basic research and animal experimentation are needed for the development of better materials and better prosthetic designs.^{19,21,24}

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30 Animal Models of Scoliosis

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I. INTRODUCTION

Spinal deformity was first described by Hippocrates.¹ Galen introduced the terms *scoliosis*, *kyphosis*, and *lordosis* into medical terminology in the second century.² It is not too much to say that the battle of research and treatment of scoliosis has been lasting for more than two thousand years. Still there remain many unknown points as to its causes and pathology in spite of many extensive experimental and clinical studies done by numerous researchers.

Experimental scoliosis has been produced using different animals since the beginning of the 20th century. The fundamental goals of experimental scoliosis are to produce ideal models of scoliosis, which are comparable to human idiopathic scoliosis and to clarify the etiology and develop new therapeutic methods. According to the literature, surgical procedures performed directly on the spine and its vicinity been used to produce scoliosis as have dietary feeding and injection of pharmacological agents. It has been a process of “trial and error” and each of the animal models has its own characteristics. In this chapter, experimental models of scoliosis from the literature are summarized, with emphasis on the experimental procedures for producing scoliosis and the effects of species, age and size of the experimental animal.

TABLE 1
Animal Selection

Procedures	Animals
Prenatal procedures	Lamb, mouse, rat, chicken egg
Immobilization using external fixation	Rat, rabbit, dog
Pinealectomy	Chicken, rat
Direct injuries of epiphysial plate	Pig, goat, dog
Resection and/or incision of ribs, transverse processes, and/or ligaments	Rabbit, pig, monkey, baboon
Rhizotomy, intercostal nerve resection, or spinal cord injuries	Rabbit, dog
Tethering	Rabbit, dog, rat
Excision of paravertebral muscle	Rabbit, rat, mouse, monkey
Electrostimulation	Rabbit, dog
Irradiation	Rabbit
Magnet implantation	Rat

II. ANIMAL SELECTION

Many animal species including mammals and birds have been used (Table I). Rabbits and rats are selected most frequently among mammals. Dogs, pigs, and monkeys also have been used. However, most of them are quadrupedal except monkeys. In these animals, the anatomic structure of the spine is different from that of human. Therefore, the models in quadrupedal mammals have certain limits for researching the etiology of scoliosis that seem to be influenced by gravity. For this reason, Yamamoto³ and Machida⁴ produced scoliosis in bipedal rats using Goff's method⁵ and studied the effect of gravity on the occurrence of scoliosis. Birds are bipedal animals and better suited for these circumstances. Chickens have been reported to develop spontaneous scoliosis without any congenital vertebral anomalies.⁶ Chickens have also been used to produce scoliosis by pinealectomy because the location of the pineal gland can be easily approached.⁷⁻¹⁵ However, the chicken spine has no intervertebral discs and tends to fuse spontaneously with only two levels remaining unfused in the thoracic portion. This anatomic difference from humans limits the use of the model for investigating the etiology of scoliosis. In addition to the anatomic features, age and size of the animal are also very important because the onset and progression of scoliosis are strongly related to growth. Each animal species has its own rate of growth. For most studies young animals are selected, such as 1-10-week-old rabbits, 2-20-week-old cats, or 6-20-week-old dogs.

III. ANIMAL MODELS OF SCOLIOSIS

Animal models of scoliosis are divided into two groups, spontaneous and induced scoliosis (Table 2). Several species of chicken,⁶ quail,¹⁶ duck,¹⁷ rat,¹⁸ rabbit^{19,20} and horse²¹ has been reported to show spontaneous occurrence of scoliosis, although congenital vertebral anomalies were involved in some of them. Experimental methods for induction of scoliosis in the prenatal periods include maternal exposure to reduced oxygen (hypoxia) in mice,²² and injection of insulin,²³ 6-aminonicotinamide,²⁴ azaserine,²⁴ or thiadiazole²⁴ into chicken eggs. Kent and Zingg²⁵ also reported surgical procedures through a hysterotomy in a lamb. Most methods of creating experimental scoliosis involve induction during the postnatal period. Although many procedures have been reported, they are mainly classified into dietary deficiency, immobilization, systemic procedures (such as pinealectomy), and local procedures (Table 2). Local procedures are those applied directly to the (1) spinal column such as unilateral damage of the epiphysis by irradiation^{26,27} or surgical resection,^{28,29} unilateral resection of the transverse processes and laminae,³⁰ and unilateral fusion of laminae and transverse processes;^{31,32} (2) the surrounding tissues, such as unilateral resections

TABLE 2
Types of Experimental Scoliosis

- I. Spontaneous occurrence
 - II. Artificial production
 - A. Prenatal induction (hypoxia, hypovitaminosis, insulin injection, etc.)
 - B. Postnatal induction
 - 1. Dietary deficiency (lathyrism, etc.)
 - 2. Immobilization (cast, band, or splint)
 - 3. Systemic procedures (such as pinealectomy)
 - 4. Local procedures
 - Damage to spine (epiphysis, lamina, spinous or transverse process)
 - Damage to surrounding tissues (ligament, rib, or muscle)
 - Damage to neural tissues (spinal cord, root, or intercostal nerve)
-

of transverse ligaments,^{30,33} ribs^{30,34–40} and paravertebral muscles;^{30,34,41–44} and (3) neural tissues, such as surgical damage of spinal cord, roots and intercostal nerves.^{45–51}

A. DIETARY METHODS

Experimental diets containing *Lathyrus odoratus* induced scoliosis.^{3,52,53} Geiger et al.⁵² investigated the effect of feeding lathyrus peas and succeeded in inducing lathyrism in young and adult rats. He noticed growth retardation of young animals with other symptoms, such as lameness, spinal curvature, sternal curvature, etc. Autopsy showed extreme curvature that was mostly ventral in the thoracic region. Ponseti⁵³ studied characteristics of scoliosis induced by lathyrism in rats to explore the pathogenesis of scoliosis and reported hitherto unrecognized lesions in the epiphyseal plates. Yamamoto² fed bipedal rats on modified Steenbock's diet that included *Lathyrus odoratus* to analyze the influence of the upright position on the development of scoliotic deformity due to dietary feedings. He compared them with rachitic quadrupedal, control bipedal, and control quadrupedal rats, and reported that marked scoliosis developed only in rachitic bipedal rats. This result indicated that the upright position played an important role to produce marked scoliosis even in rachitic rats. Compared with the relatively large amount of *Lathyrus odoratus* that was needed to produce scoliosis, feeding a small amount of B-aminopropionitrile (BAPN) has also been proved to produce scoliosis and other skeletal deformities by Lalic and Angevine.⁵⁴

Vitamin deficiency is another way of dietary feeding to produce scoliosis in animals.^{55,56} Kitamura et al.⁵⁵ reported that rainbow trout developed scoliosis or lordosis during feeding a synthetic diet with a lack of vitamin C. Lim and Lovell⁵⁶ studied the effects of dietary feeding with vitamin C deficiency on the spinal columns of channel catfish that presented scoliosis or lordosis with the frequency of 60.9% 22 weeks after feeding.

B. IMMOBILIZATION

Immobilization has proven to be one of the ways that induces scoliosis in animals. Cast, strip, and splint were reported as tools to immobilize animals into a scoliotic position.^{57–59} Wullstein⁵⁹ first reported experimental scoliosis in two dogs provoked by immobilizing them into a scoliotic position with strips of leather for seven to 13 months. The scoliosis produced in his study was slight — a long C curve involving the whole spine. Hakkarainen⁵⁷ used a three-point plaster-of-Paris corset with a thin layer of foam rubber for immobilizing rabbits in a scoliotic position for two to 5 weeks. He observed regression of scoliosis less than 30 degrees at the time of removal of immobilization, whereas progression of scoliosis occurred when the initial curve exceeded

30 degrees. Poussa et al.⁵⁸ investigated the effect of forced lordotic position by fitting extension splints in rabbits. Their study was based on the concept of rotated lordosis in the pathological mechanism of scoliosis introduced by Somerville,⁶⁰ Roaf,⁶⁰⁻⁶² and Dickson.⁶³ and 53.5% of rabbits used in this experiment demonstrated scoliosis greater than 10 degrees with a mean of 47 degrees. An external fixation apparatus developed by Stokes, et al.⁶⁴ was another device to produce scoliosis due to vertebral wedging. Mente et al.⁶⁵ applied it to rat tails for establishing if scoliosis progression could be explained in terms of mechanical forces causing vertebral wedging. Not only did they produce vertebral wedging and scoliosis, but also they showed the reversal of an induced vertebral wedging by distraction instead of compression.⁶⁶

C. SYSTEMIC METHODS

1. Pinealectomy (Figure 1)

Thillard¹⁴ was the first to report experimental scoliosis using pinealectomy. She produced scoliosis by removing the pineal glands of young chickens (2–3 days old). Dubousset et al.⁹ subsequently reported that the experimental removal of the pineal gland with its stem and part of the choroid plexus of the third ventricle in young chickens (1–5 days old) produced a high rate of severe or moderate deformity of the spine, but they failed to produce scoliosis in 20 quadrupedal rats with the same procedure. Their study also indicated that the scoliotic deformity in the chicken has the same anatomical characteristics (vertebral rotation and rib hump) as those found in human idiopathic scoliosis.

Machida et al.^{10,67} advanced Dubousset's experimental work and performed an electrophysiological study on experimental scoliosis of pinealectomized chickens. They found no pathologic change in the brain in either the pinealectomized scoliosis group or non-scoliosis group. However, the somato-evoked potential (SEP) data showed significant abnormalities in the scoliosis group. By electrophysiological examination, cortical potentials in the scoliosis group were found delayed, suggesting conduction disturbance rostral to the brain stem. Their results strongly suggested that a certain yet unidentified defect of neurotransmitters or neurohormonal systems in the pineal body must play a major role in this model.

Machida et al.^{11,12} also reported the role of the deficiency of melatonin and serotonin. They compared the occurrence and severity of scoliosis in each group treated with melatonin and serotonin. The result showed that scoliosis developed in 73% in the serotonin group, and 20% in the melatonin group. The melatonin-treated chickens with scoliosis had less severe spinal deformity than those in the serotonin-treated group. Moreover, in their serial study, they were able to prevent development of scoliosis by using 5-hydroxytryptophan (5-HTP), a precursor of serotonin, which can pass through the blood-brain barrier. They proposed that a serotonin deficit secondary to a defect of melatonin may have disturbed postural muscle tone or postural equilibrium resulting in scoliosis in the pinealectomized chicken. Consequently they implied that prevention of the development of scoliosis or its progression in chickens treated with 5-HTP suggested that serotonin may have potential therapeutic value. Finally, Machida et al.⁴ performed pinealectomy in quadrupedal and bipedal rats. They reported that the scoliosis developed only in pinealectomized bipedal rats, not in quadrupedal rats, and suggested that the bipedal condition was important for the etiology of human scoliosis.

Using the pinealectomized chicken model, Coillard et al.⁷ defined three types of morphological deformations of the vertebrae. Vertebral deformity type 1 was characterized by three-dimensional corporal torsion, which defined the horizontal disorientation of the curve. Vertebral deformities types 2 and 3 were the transitional vertebrae in the curve and defined as lateral imbalance in the election plane of the curve. They compared the result with human idiopathic scoliosis and suggested that the determination of morphoanatomical criteria was possible to force a new therapeutic strategy based on real vertebral and spinal deformation.

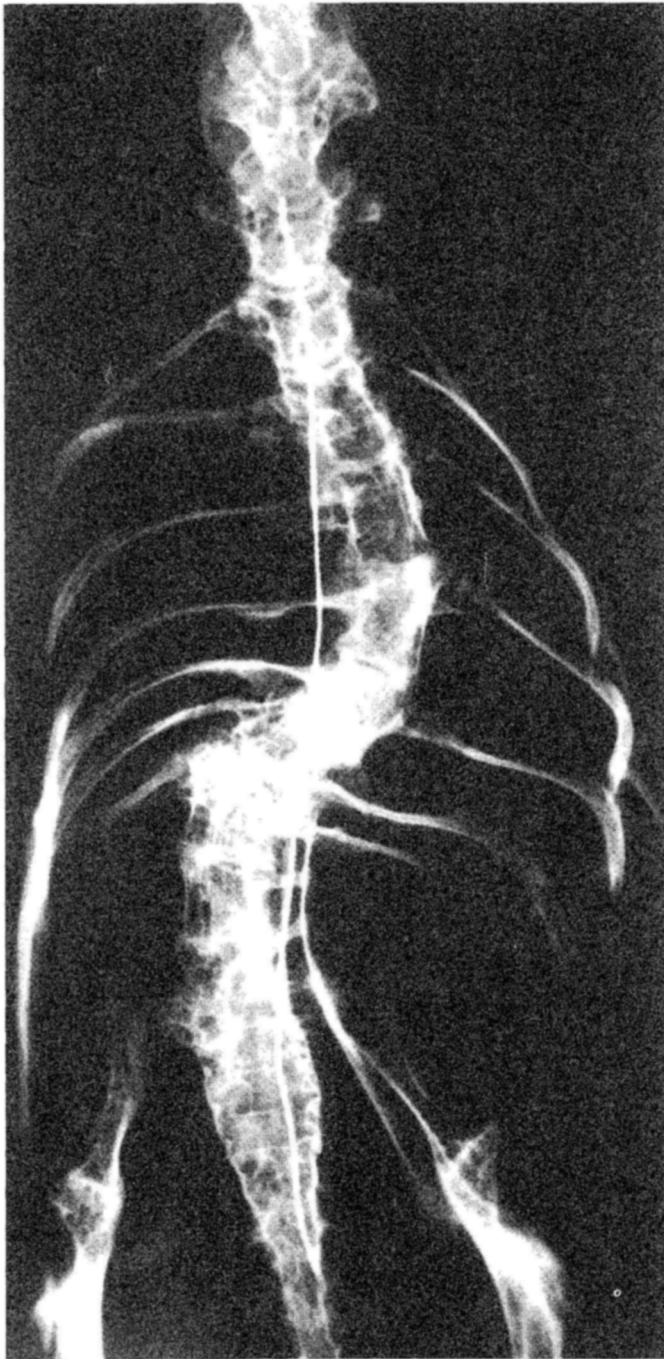


FIGURE 1. Scoliosis in a pinealectomized chicken. The pineal gland was removed at three days of age. Scoliosis developed and progressed to 83 degrees at 16 weeks of age. The wire that marks the tips of the spinous processes clearly demonstrates rotation of the vertebral column.

Wang et al.¹⁵ characterized the scoliosis produced in young chickens after pinealectomy and compared these characteristics with those seen in human adolescent idiopathic scoliosis. The results of their study showed that 60% of chickens that received pinealectomies three days after hatching acquired scoliosis. They reported that similarities included development of single and double curves,

degree of curvature, stability of the curve, numbers of vertebrae involved, direction of rotation, and progression characteristics and that differences included wedged vertebrae in the chickens, in conjunction with curve development and increased variability in vertebrae involved.

Kanemura et al.¹³ clarified the natural course of scoliosis after pinealectomies in chickens. They found that 85% of animals in the pinealectomy group developed scoliosis (7–85 degrees) featuring a three-dimensional spinal deformity consisting of both lateral curvature and vertebral rotation with rib humps. They found that the scoliotic curvature progressed as the pinealectomized chickens grew older at least until 16 weeks of age (equivalent to the age of puberty in humans). The natural course of scoliosis in the pinealectomized chickens followed one of three patterns; (1) scoliosis progressed rapidly and finally reached more than 84 degrees at the age of 16 weeks, (2) scoliosis progressed more slowly and was usually mild at the age of 16 weeks, and (3) no spinal deformity was seen during the observation period. They could predict that scoliosis exceeding 20 degrees within four weeks after pinealectomy would show progression. Their study also showed pinealectomized chickens had several other differences from normal chickens, such as poor weight gain, underdeveloped cockscombs, and the late onset of egg laying. Consequently the scoliosis developing in chickens after pinealectomy was similar to human idiopathic scoliosis and thus seems to be a useful model of idiopathic scoliosis.

D. LOCAL METHODS

1. Intercostal Nerve Resection

Intercostal nerve resection, including rhizotomy, is one of the procedures that produce scoliosis constantly. Historically, these procedures have been studied by many investigators.^{28,47–51,68} However, the developmental mechanisms of scoliosis are still controversial although these procedures are known to cause a condition of muscle imbalance.

Liszka⁴⁷ compared the effects of unilateral transaction of four anterior and posterior roots with those of five posterior roots on the spine. Both groups demonstrated scoliosis with convexity toward the affected side although rabbits with resections of five posterior spinal roots showed more marked scoliosis. He assumed that elimination of sensory impulses was more detrimental to the stability of the spinal column than motor paralysis of the muscles. MacEwen⁶⁹ repeated these experiments and reached the assumption that severance of the reflex arc was responsible for the development of scoliosis.

Alexander et al.⁴⁸ reported the effects of a number of roots transacted on the severity of scoliosis, and found that the magnitude of scoliosis depended upon the number of roots severed. They also studied the histological change of the spinal cord after the division of posterior roots. Interestingly, this study demonstrated not only dorsal column degeneration but also anterior horn cell chromatolysis in spite of preservation of anterior roots. Consequently, they concluded that scoliosis induced by rhizotomy was not due to interruption of the sensory feedback, but was caused by damage of the anterior horn cells and subsequent motor paralysis, which was contrary the assumption described by Liszka⁴⁷ and MacEwen.⁷⁰

Suk et al.⁵⁰ performed the same procedures in rabbits to confirm if scoliosis is produced by only anterior rhizotomy, posterior rhizotomy, or a combination. They found that three groups demonstrated almost the same severity of scoliosis. They concluded that scoliosis might be induced by selective posterior root paralysis as well as anterior root paralysis.

According to these investigations, the experimental scoliosis is different from human idiopathic scoliosis, and similar to paralytic scoliosis. Although the pathogenesis of scoliosis induced by intercostal nerve resection remains unclear, these animal models of scoliosis are reproducible and constant.



FIGURE 2. Scoliosis due to rib resection in a chicken. Three consecutive ribs on the right (T3, T4, and T5) were resected for a length of 3 mm just lateral to the costotransverse joint at the age of four weeks. Scoliosis of 42 degrees with the convexity to the operated side developed at 20 weeks of age.

2. Rib Resection (Figure 2)

Among unilateral operations on structures in the vicinity of the spine, posterior rib resection lateral to the transverse process is recognized as a procedure which constantly produces scoliosis.^{33,34,71,72} The rib cage has several important biomechanical functions related to the spine. It stiffens and strengthens the spine, thus providing greater resistance to displacement. The vertical stability of the thoracic spine is maintained by equal support of the ribs on both sides. Since

Bisgard⁴⁶ first reported experimental thoracogenic scoliosis, this type of experimental model has been used for investigation of the etiology and pathology of scoliosis.^{29,35,38,40,43,51} The results of these studies indicated that scoliosis might be caused by the weakness or absence of a structure on the convex side of the curve or the over activity of its antagonist on the concave side.

Pal and Bhatt³⁰ and Sevastik et al.⁴⁰ studied the mechanism of production of experimental scoliosis by rib resection in rabbits and developed the following hypothesis. After the resection of a few ribs on one side, the intact ribs on the opposite side exert more load against the vertebral column and this imbalance causes the vertebral column to move laterally and rotate. Scoliosis following rib resection is usually progressive until skeletal maturity is obtained. The more ribs that are resected, the longer the scoliosis becomes. The apex of the scoliosis is usually at the middle of the vertebrae from which the ribs are resected, but it varies to the upper or lower segment in some cases. The convexity of the curvature is always toward the operated side and the apical vertebral body is rotated toward the convex side of the curvature as it occurs in human idiopathic scoliosis.

While factors which affect the severity of scoliosis induced by rib resection have been suggested by DeRosa⁷³ and Langenskiold and Michelsson,^{33,34,71,73} Deguchi et al.^{74,75} reported the most detailed study on the relationship between the severity or progression of scoliosis and the number of ribs resected or the effect of age. The study showed that a younger age at operation and the resection of more ribs were important factors in producing severely progressive scoliosis. Interestingly, chickens that underwent rib transaction showed bone healing at the transacted sites several weeks after surgery and no scoliosis developed. Furthermore, the authors clearly showed a negative correlation between the number of healed ribs and the severity of the scoliosis. As the operation creates a gap in the bony skeleton along one side of the spine, mechanical pressure from the contralateral intact ribs may push the mobile column into the gap, and subsequent asymmetrical rib growth may fix and increase the deformity. However, when some of the operated ribs fuse, asymmetrical load transmission to the spine is reduced and progression subsequently may be slower. Therefore, to induce severe scoliosis by rib resection, a younger age at operation, the resection of more ribs, and little bone regeneration at the site of resection are important factors.

Deguchi et al.⁷⁵ also examined the alterations of the rib cage and vertebrae in the transverse plane using CT scanning. While there was a significant positive correlation between the Cobb angle and the apical vertebral rotational angle as in human idiopathic scoliosis, curvature following rib resection did not completely resemble the three-dimensional feature of human idiopathic scoliosis. The apical vertebra actually rotated to the convex side of the curvature and a rib hump developed on the concave side of the curve. The direction to which the anterior midline of the body deviated was different between human idiopathic scoliosis and experimental scoliosis by rib resection; it was to the opposite side of the convexity of the curvature in humans, which tended to be the convex side in scoliosis following rib resection. This is because rib resection is a localized surgical interference that influences only the operated area or its vicinity, which causes a distortion of the thoracic cage. The treated ribs result in asymmetric rib growth, which makes the midline of the body deviate to the side and prevents rib humping, which should have developed on the convex side from developing on the physiological side. Therefore, a rib hump developing on the concave side of the curve might be termed "pseudohump." Despite such problems, rib resection is one of the standardized procedures which constantly produce identical scoliosis.

3. Tethering between Scapula and Pelvis

From the view point of the direction of a tether to the spinal column, tethering procedures for producing scoliosis in animals were divided into two groups: a posterior tether and a unilateral tether. There was another classification of tethering such as external and internal. Some of immobilization tools,⁵⁷⁻⁵⁹ which were already described in this chapter, can be attributed to an external tether. A posterior tether which succeeded in producing scoliosis was induced by not only an

extension splint⁵⁸ as an external tether but also an approximation of the spinous process by nylon suture⁷⁶ and sutures of the three lumbar spinous processes.⁷⁷ These experiments were conducted to prove the concept of rotated lordosis in the pathological mechanisms of scoliosis.

On the other hand, a unilateral tether was reported as one of the successful methods to produce marked scoliosis such as unilateral fixation of ribs by Dacron thread,³⁵ placement of coil splint between articular processes,⁷⁸ and suture of the inferior angle of the scapula to the pelvis.^{79,80} According to the purposes of experimental scoliosis, it is important that experimental models of scoliosis need to be easily reproducible and remain intact on the spine and the tissues around the spine. From this view point, Sarwark's model may be appropriate to use in spinal research.

Sarwark et al.⁷⁹ reported an experimental model of scoliosis by suturing the inferior angle of the scapula to the pelvis at the base of the tail in 15-21 day old male SD rats. They released the tethers in 1–12 weeks and found that a minimum period of six weeks was needed for production of permanent structural curves. They also noted that the longer the period of tethering is, the more severe the scoliotic curves become. The characteristics of the scoliotic curves were long C shaped thoracolumbar curves with vertebral rotation and apical wedging. In addition to these morphological investigations, they studied the effects of the procedures on neurologic function. They found an increased incidence of a mild spasticity in the ipsilateral hindlimb with curvature greater than 40 degrees. The conclusions of this model included no direct trauma to the spine, existence of similar changes to human idiopathic scoliosis, technical easiness in producing scoliotic curvatures in a large number of animals in a relatively short period of time, and appropriate models for study of scoliosis.

4. Removal of Transverse Processes or Ligaments

As already described, rib cage deformity or rib resection was reported to disturb the balance of the spine and caused spinal deformity. A morphometric and morphological study on the thoracic cage indicated ribs played some role of transmission of considerable load from the sternum to the spinal column. The transverse processes and ligaments around them were important structures as load transmitters between ribs and the spinal column. Langenskiöld and Michelsson³⁴ paid attention to the importance of costo-transverse ligaments and succeeded in producing scoliosis by section of anterior and posterior costo-transverse ligaments at four or five levels in rabbits and pigs. However, severe progressive scoliosis was only seen in a few of 47 rabbits. They speculated this was because scar formation around the incised ligaments might have prevented the progression of scoliosis. The same procedures in pigs revealed a scoliosis of 30–45 degrees with the rate of occurrence at 90%. They concluded that posterior costo-transverse ligament was of decisive importance in its equilibrium and symmetrical growth by transmitting the effect of the normal muscle tone to the spine. Karaharju³⁵ also severed dorsal ligaments of the 8th to 12th ribs in three pigs and produced scoliosis of 12–35 degrees.

Pal et al.³⁰ studied the route of load transmission from ribs to the vertebral body. He pointed out the various sites, such as ribs, costo-transverse joints, transverse process, lamina, and facet joints that have possibilities in producing asymmetry in load transmission. Because posterior rib resection and hemilaminectomy have already been proved to produce scoliosis in animals, they removed unilateral transverse process and facet joints without interference of ribs in rabbits. The resection of the transverse processes from T3 to T6 showed no scoliosis or mild scoliosis. The extended resection of them from T3 to T9 also demonstrated mild scoliosis. However, removal of seven unilateral segments of the transverse processes in addition to the successive four segments of the facet joints revealed rapidly progressive lordoscoliosis with the convexity toward the operated side. The model described by Pal et al.³⁰ has proved that the mechanical asymmetry in load transmission from the ribs to the spine resulted in scoliosis, although Smith and Dickson⁸⁰ reported that the previous experiments of rib resection to produce scoliosis caused spinal cord damage due

to damages of segmental vessels at the time of rib resection and that scoliosis was induced by the spinal cord damage.

5. Muscle Imbalance

Since Arnd⁴¹ first reported the production of scoliosis in rabbits by unilateral excision of the deep back muscles, several studies dealing with muscle imbalance due to incision or excision of the back muscles was conducted to produce scoliosis in animals.^{30,33,41–44} Because Arnd's results showed scoliosis with convexity of the curve toward the unoperated side, he suggested the importance of contracture of the scar tissues for the induction of scoliosis. On the contrary, Schwartzmann and Miles⁴³ studied the effects of muscle imbalance on the alignment of the vertebral column by excision of muscles in rats and mice. They found that muscle imbalance produced not only by excision of unilateral back muscles but also unilateral muscle release with inert materials to prevent muscle reattachment caused scoliosis experimentally. They stressed the importance of muscle imbalance for producing scoliosis through the results of failure by muscle excision and release that did not produce imbalance. Furthermore, Miles⁴² advanced this experiment and found the difference of curve patterns between muscle resection and nerve resection.

Stilwell⁴⁴ used monkeys to study radiographic and histological changes of vertebrae during curve progression produced by resection of one or both sacrospinalis muscles from the sacrum to the upper thorax. All of 11 monkeys that underwent this procedure showed marked scoliosis with intervertebral disc distortion and vertebral wedging. However, his procedures included incision of the interspinous and flaval ligaments because his main concerns were not the effects of muscle excision on the spine but the mechanisms of bone modeling in a weight bearing spine under conditions of scoliosis progression. In an additional study, he failed to produce significant structural deformity or persistent curve by denervation or resection of a variety of asymmetrical muscles.

As already noted, muscle imbalance induced by unilateral excision of the back muscle or by denervation due to rhizotomy or intercostal nerve resection has been recognized to cause scoliosis in animals. Clinically, it is well known that neuromuscular disorders have many features including scoliosis. On the basis of these findings, some attempts at electrical stimulation of unilateral back muscles in animals have been done since 1970s.^{81–84} Olsen et al.⁸³ reported production of scoliosis of 6.8 degrees with its concavity toward the stimulated side by stimulating unilateral paravertebral muscles through implanted electrodes in dogs.

Monticelli et al.⁸² produced thoracolumbar scoliosis of 15–36 degrees by almost the same procedures in rabbits. Joe⁸⁴ studied the changes of muscles caused by electrical stimulation histologically. He noted that the diameter of type I fibers increased on the stimulated side, which might be the cause of muscle imbalance. These changes returned to the same level as that of the control groups three weeks after the termination of the stimulation although scoliosis remained almost the same as before. These experimental attempts generally produced mild scoliosis in animals and seemed to have some limits for producing severe progressive scoliosis.

6. Other Methods

Besides the common procedures which produce scoliosis in animals described above, several other experimental models have been reported in the literature.^{85,86} Ehara et al.⁸⁵ implanted a few magnets on the unilateral sides of the lumbar spines and succeeded in producing scoliosis in rats. Scoliosis induced by three magnets implanted tended to progress more severely than scoliosis with two magnets, with their average Cobb angles 24 degrees, and 14.2 degrees, respectively. Their final goals are clinical application for the treatment of scoliosis. However, the use of magnets basically needs further work for toxicity determination.

An interesting study has been presented by Chuma et al.⁸⁶ The development of MRI makes clear that some scoliotic patients have a spinal cord lesion, syringomyelia. It seems that scoliosis

is strongly related to syringomyelia, while the pathogenesis of scoliosis due to syringomyelia is still unclear. However, Chuma reported experimental models of scoliosis due to syringomyelia which was produced by a kaoline injection into the cisterna magna in dogs. In spite of the relatively low rate of occurrence of scoliosis (3/11) this study may have just opened the door to a new world of experimental research on scoliosis, and pinealectomy.

IV. THE USE OF SCOLIOSIS MODELS IN SPINAL RESEARCH

A. PATHOGENESIS OF SCOLIOSIS

Scoliosis resulted from a wide variety of pathological conditions that have been classified by the Scoliosis Research Society.⁸⁷ However, sixty or seventy percent of scoliosis cases are diagnosed as idiopathic scoliosis. Because of this, one of the main purposes of the experiments for production of scoliosis in animals, in which many investigators have been deeply involved as mentioned above, was to elucidate real pathogenesis and mechanisms of development of human idiopathic scoliosis. Those animal models were evaluated and compared with human idiopathic scoliosis not only morphologically by X ray or dissection, but also biochemically. The existence of rotation associated with lateral curvature and apical vertebral wedging were main concerns in morphological study. Histology observations about bone remodeling, epiphyseal changes, the spinal cord damage, or difference between convex and concave side of the paravertebral muscle were also reported in each experimental model. As for biochemical analysis in experimental animal models, some research has been done by using human blood, urine, or intervertebral discs resected at surgery.⁸⁸ Machida et al.¹¹ estimated serum melatonin in the control group and the pinealectomized group in chickens for investigating the relation between melatonin and scoliosis.

B. EXPLORATION FOR NEW TREATMENT

The other purpose of the experiments of producing scoliosis models in animals was for providing new methods of treatment of already established scoliosis. Some of the experiments were done to evaluate the influences of the same procedures as those which induced scoliosis on the opposite side of the spine.^{31,34} Others were conducted to determine whether some new methods of treatment of scoliosis were effective or not (Table 3).^{8,39,57,85,89-92} Because surgical correction of scoliosis needs direct approach to the spine, it is necessary to keep the spine and its vicinity intact at the time of producing scoliosis. From this view point, the models produced by the tethering of the scapula to the ipsilateral pelvis or pinealectomy may be ideal for searching a new procedure for treatment of scoliosis, as reported by Dabney et al.,⁸⁹ Salzman et al.,⁹³ Glassman et al.⁹⁰ and Deguchi et al.⁸

V. FUTURE DIRECTIONS OF RESEARCH

Most of the experiments related to the animal models of scoliosis were conducted early in this century by analyzing morphological changes of the spine due to each procedure. Because of limitations of the methodological approach, such as with X ray or histology at that time, it was always controversial to make a conclusion as to whether the results obtained from the experiments were primary or secondary. On the other hand, there were some experiments whose data contradicted each other in spite of use of almost the same procedures. This kind of contradiction may be possibly happen because development of idiopathic scoliosis is evoked by more than one etiologic factor as Ponseti et al.⁵³ described. Thus, it is now widely accepted that more than one pathological mechanism is responsible for idiopathic scoliosis and that idiopathic scoliosis is a group of disorders that show some common parameters. From this viewpoint, many original ideas and experiments using animal models will be reported and studied in the future. It cannot be definitely said in which

TABLE 3
The Use of Scoliosis Models in Spinal Research

Procedures used for induction of scoliosis	Procedures used for correction of scoliosis	First author, year ^{Ref.}
Placement of a staple on the lumbar spine and intercostal muscle	Staple on the opposite side	Nachlas 1951 ³¹
Hemilaminectomy, incision of ligaments	Cut of the posterior and anterior costo-transverse ligament on the concave side	Langenskiöld 1962 ³⁴
Immobilization by cast	Soft tissue release on the concave side	Hakkarainen 1981 ⁵⁷
Tethering of the scapula to the ipsilateral pelvis with suture	Distraction with spinal instrumentation	Dabney 1988 ⁸⁹ , Salzman 1991 ⁹² Glassman 1995 ⁹⁰
Resection of three intercostal nerves	Rib shortening on the concave side	Sevastik 1990 ³⁹
Unilateral removal of back muscles	Direct attachment of two or three magnets on concave side of the lumbar spine	Ehara 1992 ⁸⁵
Unilateral resection of five ribs	Spinal instrumentation (shape memory alloys)	Sanders 1993 ⁹¹
Pinelectomy	Rib resection on the concave side of the scoliotic spine	Deguchi 1996, 1997 ^{8,75}

direction experimental research of scoliosis will advance toward in the future. However, there are three or four possible clues for the future experimental research of scoliosis right now.

First, as three dimensional analysis by computers is becoming popular in clinical research of idiopathic scoliosis, experimental works will be conducted for the proof of hypothesis or results that are derived from biomechanical analysis by computers, and vice versa. Scoliosis can be said to be a disorder of misalignment of vertebrae happening during the growth period. Thus, a biomechanical approach for experimental scoliosis is absolutely important with the help of computer analysis.

Second, biochemical analysis of experimental scoliosis will be advanced further. Biotechnological skills and machines have developed recently and will continue to develop more in the future. These developments will make microanalysis of hormones and other substances possible in the blood, urine, and tissues not only *in vitro* but also *in vivo*. Pathogenesis of scoliosis in pinealectomized chickens will be studied continuously biochemically and may open the door to a new treatment of human idiopathic scoliosis without braces or surgical operations.

Third, a gene analysis will be applied for the study of the experimental scoliosis in animals. Particularly, spontaneous occurrence of scoliosis in a highly inbred line of chickens reported by Taylor⁶ and in Ishibashi rats,¹⁸ may be a good pathology for a gene analysis. On the other hand, it may be possible to produce experimental models of Recklinghausen disease, Marfan syndrome, etc., which are recognized as hereditary diseases by using the method of genetic recombination.

Finally, the studies and analysis of new surgical or physiological treatments using some kinds of the animal models of scoliosis will be continued in the same trend as those reported in the literature.

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Part VIII

Microsurgical Technique



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31 Microsurgery and Orthopedic Animal Models

Yuehuei H. An

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I. INTRODUCTION

The microsurgical technique has been well developed for more than 20 years. The “dawning of microsurgery” in the early 1960s arose from the development of the operating microscope,¹ the availability of heparin,² the perfection of microinstruments, the previous work on vessel anastomoses and replantation and transplantation of animal and human organs.

From the mid 1950s to the early 1960s several groups in Russia,³ the United States,⁴ and Japan,⁵ engaged in the investigation of replanting animal legs. Due to the lack of established microsurgical techniques, there were more failures than successes. By the early 1960s, successful replantation of canine legs had been reported. With the development of microinstruments and the use of the microscope, especially after the availability of a new microvascular method by Jacobson and Suarez

in 1960,⁶ the era of microsurgery began. Using the method by Jacobson and Suarez, vessels 1 mm in diameter could be anastomosed. In 1961, Lee and Fisher published their first paper on the portacaval shunt in the rat.⁷

In May 1962, Malt and McKhann in Boston achieved the world's first arm replantation which was reported in *JAMA* in 1964.⁸ In January 1963, Zhong-Wei Chen et al. in Shanghai successfully reimplanted a complete amputation of the forearm which was reported in *Chinese Med. J.*⁹ In 1963, Inoue et al. succeeded in replanting a severed hand.¹⁰ In July 1965, Komatsu and Tamai achieved a replantation of an amputated thumb.¹¹ In Shanghai, Chen and Yang in 1967 first performed successful toe-to-hand transfer.^{12,13} Similar work was first reported in English literature by Cobbett in 1969.¹⁴

From the mid 1960s to the early 1970s, more achievements were reported in experimental microsurgery. The first model of free skin flap in the dog was reported by Krizek et al. in 1965.¹⁵ The first muscle transfer in the dog was reported in 1968¹⁶ and vascularized bone graft by Buncke et al. in 1967.¹⁷ By the early 1970s, Lee's group had established numerous rat models of organ transplantation.¹⁸

In 1973, the first human application of free skin flap was reported by Daniel and Taylor¹⁹, and the first case of a pectoralis major muscle transplantation was done by a Shanghai group.²⁰ Also in 1973 (published in 1983), Ueba and Fujikawa successfully transferred a vascularized fibular bone graft for the treatment of neurofibromatosis of the ulna.²¹ Following this were reports on free vascularized fibular graft transfer to tibial defects by Taylor et al.,²² and free groin-iliac osteocutaneous flap by Taylor and Watson,²³ and Tamai.²⁴ In 1976, Baudet et al. proposed the term *musculocutaneous flap* and emphasized the usefulness of the latissimus dorsi musculocutaneous flap.²⁵

By the late 1970s, the development of microsurgery had already come into a mature stage. Clearly, the current microsurgical techniques depend mostly on the extensive research work using animal models.

II. TRAINING OF MICROSURGICAL TECHNIQUES

Self confidence is the key in learning microsurgical technique. Most beginners are able to complete a successful vascular anastomosis with 1-3 days of training in the laboratory. The learner has to realize that it is a hard and continuous training process with an initial intensive exercise period of six hours per day five days per week for at least four weeks and refreshment exercises (several anastomoses per time) several times per year.

Proper training in microsurgical techniques could not be done without using animals, for no other alternatives can mimic a living vessel which possesses a contractible wall and the ability to clot. However, well prepared beginners will need fewer animals to complete their training, for they have practiced by stitching rubber gloves or silicone tubes and anastomosing vessels harvested from commercially available meat.²⁶ Another training model was reported by Kim et al., which uses cold stored vessels harvested from sacrificed animals used in other projects.²⁷

Although rabbits and cats have been used, rats have been the dominant animal subject at most training centers. Rats are anesthetized by intraperitoneal injection of pentobarbital. Rat abdominal aorta and carotid arteries are the first choice for a beginner because they are larger and stiffer than the femoral artery. They are easier for beginners to get the first functioning anastomoses, which is very important for building up confidence for mastering the technique. Also these vessels are located deep in the body, and require more work to expose them. Therefore, techniques of microdissecting of tissues, ligating vessel branches, and tying knots can be exercised before the finer vascular work. For a careful and patient operator, a carotid artery of a 400 to 500 gm SD rat could be cut and anastomosed up to eight times.

Rat femoral vessels are the second choice for practice. They are smaller than the aorta and carotid vessels, and they contain thinner vessel walls. One needs at least several days practice on

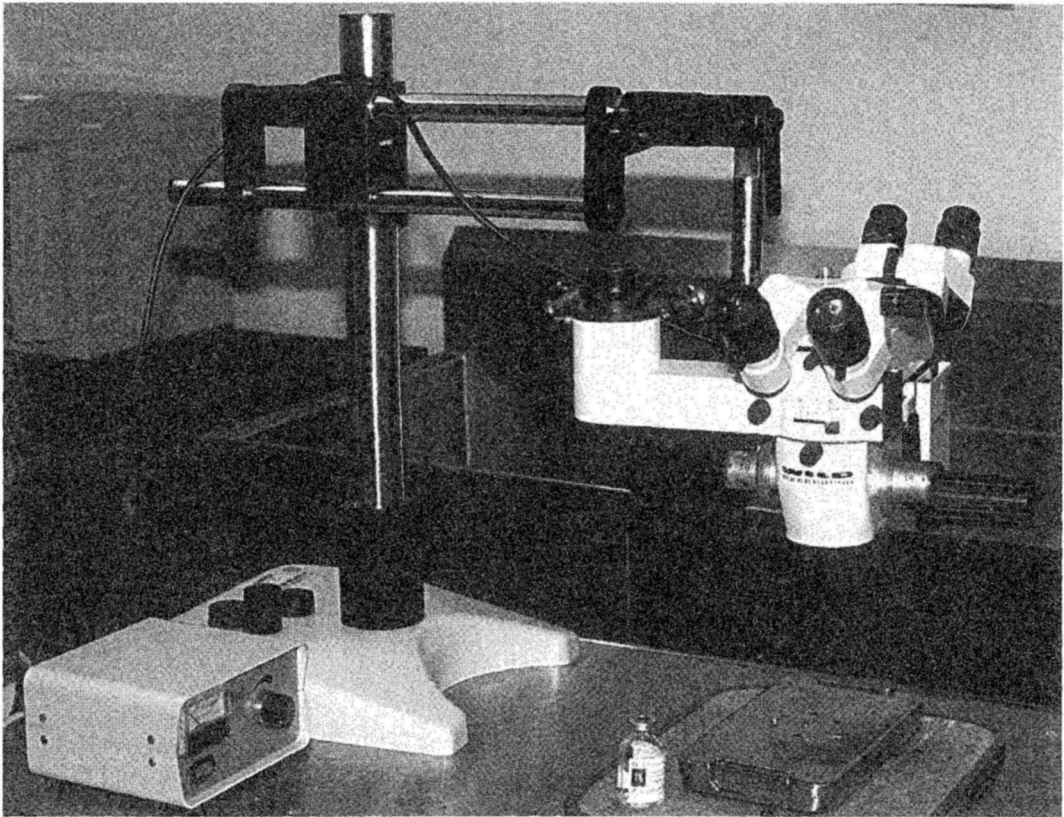


FIGURE 1. A Wild (Type 308795, Heerbrugg, Switzerland) table top operating microscope.

aorta or carotid artery before working on a femoral vessel. Toward the end of a training program, one may want to repair a rat tail vessel.²⁸ However, the femoral and tail arteries are not the first choice for beginners.

Also, exercise on vein anastomoses is better started at the later stage of the training program because it is more challenging than arterial repair.

III. BASIC REQUIREMENTS FOR EXPERIMENTAL MICROSURGERY

A genuine scientific interest, a positive attitude, responsibility and patience are essential for the surgeon performing the surgery. All personnel should be well prepared and highly motivated. Team work is important for surgical performance as is collaborating with scientists of other specialties.

A normal operating room for animal surgery should be equipped with an operating microscope. A steady and adjustable operating table is ideal. The housing facility needs to be equipped with intensive monitoring devices such as a skin thermometer and a Doppler vessel blood flow monitor. A qualified full-time technical assistant is necessary to achieve optimal postoperative care and monitoring of the animals.

A table top microscope is economical and sufficient for most procedures on rats and rabbits (Figure 1). Only a simple and personalized set of microinstruments is necessary. This set should include two microsurgical forceps, a vessel dilator, a needle holder, dissecting scissors, straight scissors, several single vessel clamps and a vessel approximator (twin clamps). Proper maintenance of instruments is necessary (Figure 2).

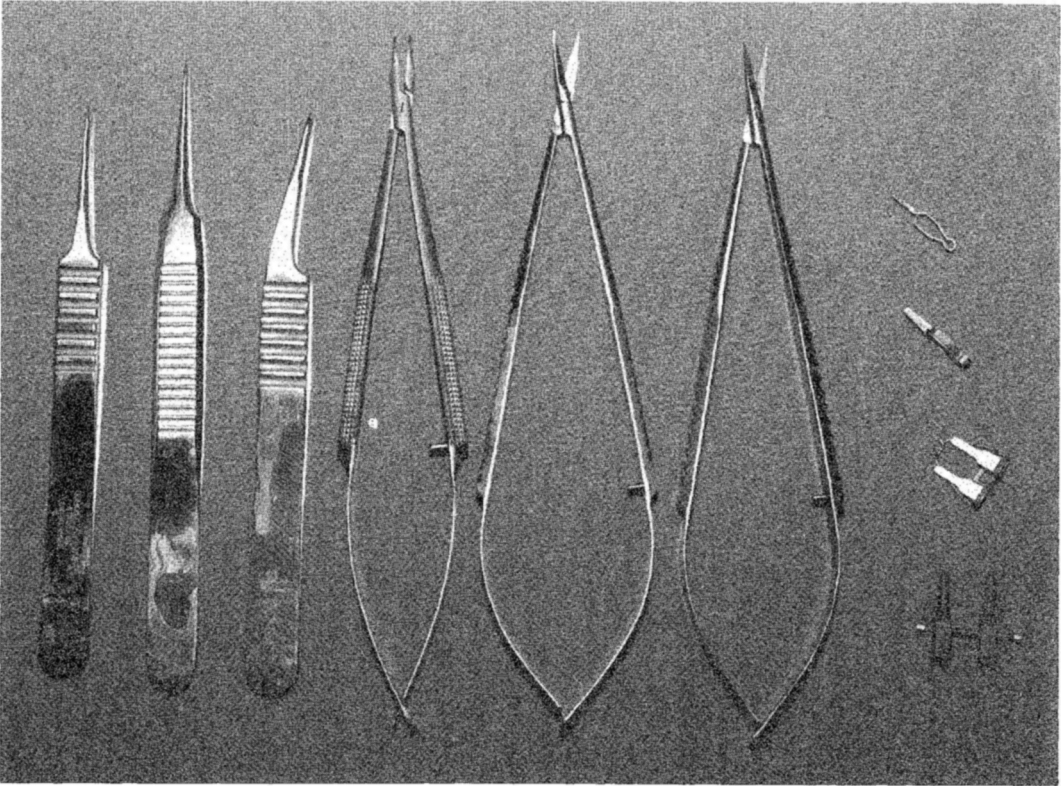


FIGURE 2. Essential microsurgical instruments (left to right): two microsurgical forceps, a vessel dilator, a needle holder, dissecting scissors, straight scissors, two single vessel clamps, and a vessel approximator (twin clamps).

IV. BASIC TECHNIQUES

Microsurgical techniques include micromanipulation of tissues, microdissecting, and microsuturing techniques for different types of tissues such as vessels and nerves. For the purpose of this book, only the suturing techniques for vessel and nerve repair are described. One should read the books by Lee,¹⁸ Pho,¹² and Mehdorn and Müller²⁹ for more details.

A. VESSEL ANASTOMOSIS

1. End-to-End Anastomosis

End-to-end anastomosis is the conventional technique for repairing both small arteries and veins.^{12,18,29,30} After the artery is freed of surrounding soft tissues and branches ligated, a vessel approximator is applied before the cut is made with a pair of straight scissors. A light colored background plastic sheet (commonly yellow) could be used underneath the vessel to give a sharper and clearer view of the field. The loose soft tissue around the end of vessel can be trimmed off using the “pull-and-cut” method (Figure 3A). Spasm of the vessel opening is common which could be solved by warm saline irrigation or by using a vessel dilator (Figure 3B,C). For bigger vessels such as abdominal aorta or carotid artery, a curved needle holder is more efficient for dilating the vessel opening (Figure 3C).

If a carotid artery is used for anastomoses, 8–10 stitches should be placed in the sequence shown in Figure 4A. For the first and second sutures a tail is needed for retraction. There are

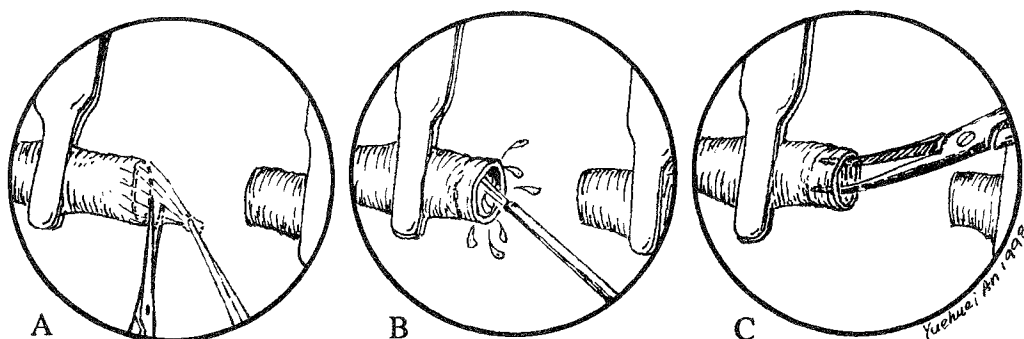


FIGURE 3. The procedure of trimming loose connective tissues around the vessel opening (A), saline irrigation (B), and vessel dilation with a curved needle holder (C).

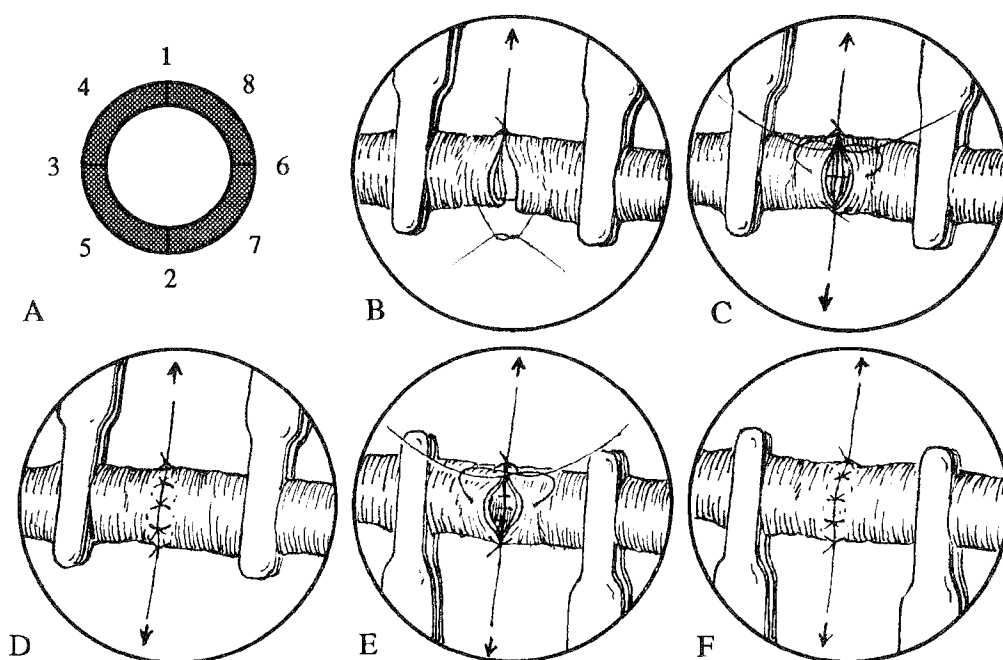


FIGURE 4. Procedures of end-to-end anastomoses of small vessels: (A) the sequence of suture placement; (B) the 1st and 2nd sutures; (C) the 3rd suture; (D) the 4th and 5th sutures (the front side is complete now); (E) the 6th suture after the vessel-clamps assembly is flipped 180°; and (F) the 7th and 8th sutures (the anastomosis is completed).

commercially available weights for use in retraction, but a single heavier vessel clamp will serve the same purpose (Figure 4B,C). After one side of the vessel is sutured the whole assembly, including the vessel and the clamps, will be turned over to expose the other side of the vessel for completion of the anastomoses (Figure 4D,E). For testing the patency of the anastomoses, a full and pulsating artery is the best test. For vein repair, the milking test could be used (Figure 5). However, it is not recommended in clinical settings to use this test for its potential traumatic effect. Minor leaks are often seen after releasing the vessel clamps. A cotton ball can be applied to the anastomotic site for a short period (3–10 min.), thus stopping most leaks.

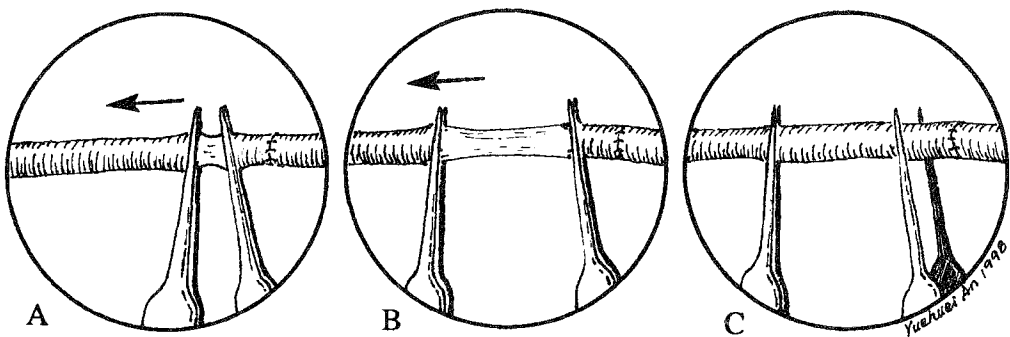


FIGURE 5. A milking test for testing vessel patency.

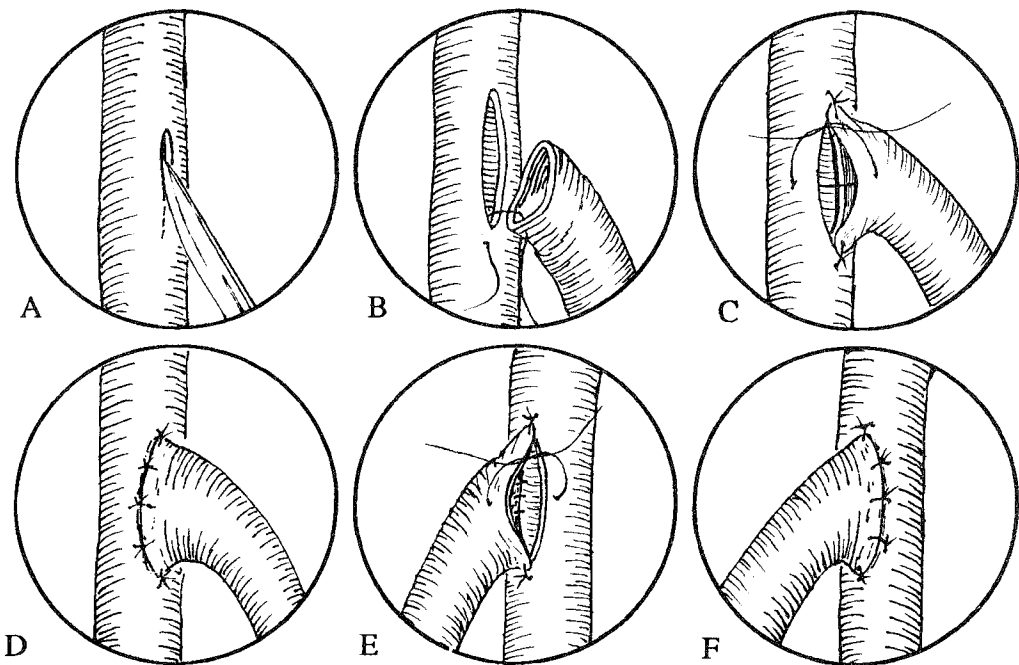


FIGURE 6. Procedures of end-to-side anastomoses of small vessels: (A) A longitudinal incision is made using a small knife. (B,C) Two angle stay sutures are placed to connect the two vessels and the 3rd suture is placed on the front side. (D) The 4th and 5th sutures are used to complete the front side. (E,F) The whole assembly is flipped 180° to the right side for suturing the back side.

2. End-to-Side Anastomosis

Compared to end-to-end technique, end-to-side anastomosis is much less used and mainly for one-vessel recipient area or for overcoming unequal vessel size. This can be practiced on various vessels such as arterio-arterial or arterio-venous anastomoses. Basically, a longitudinal incision is made using a small knife and extended with scissors. Two angle stay sutures are placed to connect the two vessels. Finally, the anterior and posterior walls are sutured (Figure 6).

3. Telescoping or Sleeve Technique

Lauritzen introduced the telescoping or sleeve technique for microvascular repair in 1978.³¹ It is used when the upstream vessel is smaller than the downstream vessel. For example, in an artery repair, a smaller proximal end (donor artery) is inserted in the distal artery (recipient artery). An artery cuff technique described by Hung et al. was based on the same principle.³² Saitoh et al. found that this technique gave very high patency rates when used at both ends of vein grafts interposed for venous defects. No difference was noted when comparing it to conventional end-to-end technique in the degree of stenosis.³³ Further modifications of this technique include a “sutureless sleeve,”³⁴ a three-stitch sleeve,³⁵ and various other sleeve methods.³⁶

4. Precautions

Factors affecting success of vessel repair include stitching technique, tension at the anastomotic site, excessive injury of the vessels openings by careless practice, or thermal conditions (should be kept close to 37° C). A careful and protective manner is essential for successful anastomoses. Identical stitch intervals are more important than the number of stitches. No more than 10 stitches for rat aorta or carotid artery and eight for femoral artery are necessary.³⁷ Steady and firm penetration of the vessel wall, using a proper size needle, is essential for avoiding unnecessary damage and leaking. Operators should do their best to avoid introducing any adventitia, loose fibrin, or foreign materials into the vessel lumen. Excessive tension can be solved by freeing more soft tissues around the vessel or by using a vessel graft when necessary. A reasonable thermal condition can be maintained by using warm saline irrigation or sponges soaked with warm saline, keeping the whole anastomotic procedure “under water.”

B. NERVE REPAIR

1. Basic Anatomy

A peripheral nerve is composed of one or more fascicles, covered by an epineurium (Figure 7). Each fascicle is formed by a conglomeration of thousands nerve fibers (axon with surrounding Schwann cell sheath). The connective tissue surrounding the fascicle is called perineurium. A mesoneurium is attached to one side of the nerve trunk for blood supply of the nerve.

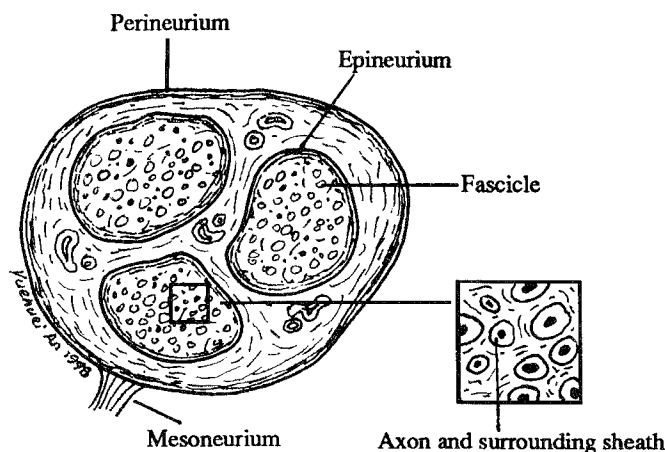


FIGURE 7. Transverse section of a peripheral nerve.

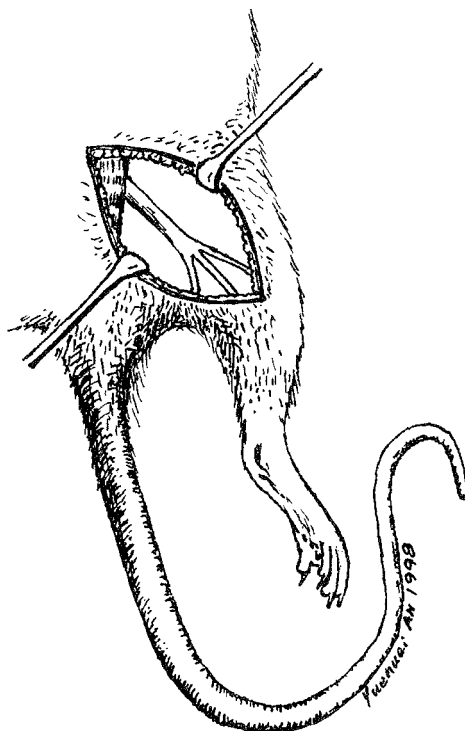


FIGURE 8. Rat sciatic nerve is made up of three fascicles

2. Suture Materials

The rule is that the finest possible suture material should be used in the smallest possible quantity. In most cases, 9/0, 10/0 or 11/0 monofilament nylon threads on tapered needles should be used. A newer product, polylactic acid suture, can make remarkable union of the severed nerve with no inflammatory reaction upon absorption of the sutures.

3. Animal Selection

Rat sciatic nerve, 1.0 mm in diameter (for rats with 400 gm body weight), runs behind the biceps femoris and divides into tibial, peroneal, and sural nerves when it reaches the knee level (Figure 8). The sciatic nerve is made up of three fascicles which enables both epineural and perineural anastomoses (Figure 9). Rabbit sciatic nerve is much thicker, 2.5–3.0 mm in diameter (for rabbits with 4 kg body weight) and is longer than rat sciatic nerve. It also runs behind the biceps femoris and divides into tibial, peroneal, and sural nerves at the knee level. Rabbit sciatic nerve is easier for vascularized nerve graft.³⁸

4. Types of Repair: Epineural or Perineural

Epineural repair means the sutures are placed into the circumference of the nerve (epineurium) following the basic principles of microsurgery as for vessel anastomoses. For a peripheral nerve of 1-2 mm in diameter, 4–6 stitches are sufficient (Figure 9B). If the fascicles or funiculi in the nerve can be dissected clear and exact adaptations can be achieved, a perineural suture technique

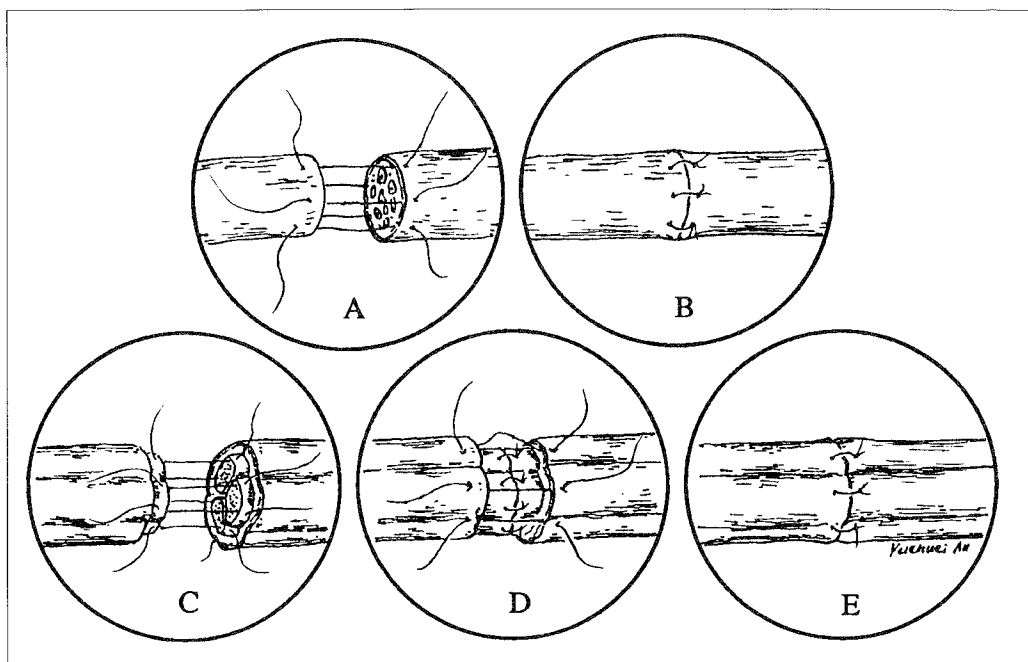


FIGURE 9. Epineural (B) and perineural (C) anastomoses.

(or interfascicular or funicular suture technique) should be used (Figure 9C). Two or three stitches are sufficient for each fascicle. After the completion of interfascicular repair, the epineural sheath should be sutured interruptedly to ensure a tension free repair.

The interfascicular suture technique was first introduced for clinical application by Smith in New York³⁹ in 1964 and Ito et al.⁴⁰ in Japan also in 1964 (according to Tamai¹³). Few years later, the work by Bora⁴¹ and Hakstian⁴² also contributed in the fascicular orientation and repair. Reported by Millesi et al., interfascicular nerve grafting can produce recovery of useful function in 80% of their patients.⁴³

5. Precautions

The first step of nerve repair is exposure of a nerve lying in scar tissue (neurolysis). Repair of the nerve should not be started unless the corresponding fascicles and/or group of fascicles have been identified in the best possible way. Tension should be avoided because the greater tension the more scar tissue will form. Tension also reduces the precision of fascicle adaptation. The mesoneurium must be freed enough to obtain an adequate length of the nerve for repair. If a segment of the nerve is resected, a nerve graft should be considered.

V. MICROSURGERY AND ORTHOPEDIC ANIMAL MODELS

A. REIMPLANTATION AND TRANSPLANTATION OF SEVERED LIMBS

Most clinical achievements in microsurgery were based on animal research.⁴⁴ In the late 1950s in Japan, Tamai's group tried to replant incompletely amputated thighs in two human cases.¹³ Due to the lack of microsurgical technique, the results were unfavorable. In 1961 they started extensive

animal research, working on replantation of canine thighs and succeeded after two years of hard work.⁵ At the same time or earlier, two other groups, Lapchinsky in Russia³ and Snyder et al.⁴ in the United States reported their results on canine thigh replantation. Due to the technique of vessel anastomoses and the use of an operating microscope, the dream of replanting severed limbs finally became a reality in 1962.⁸

An early report of animal study of digital replantation was done by Buncke et al. in 1965.⁴⁵ Most of these cases failed. A successful replantation of a totally amputated thumb in human had already been achieved in 1965 by Komatsu and Tamai.¹¹ Today, digital replantation has become a common procedure in many major centers. Cheng replanted 304 severed digits from 1978 to 1983 with 280 survivals (92.1%).⁴⁶ Cheng once treated a case of traumatic amputation of all ten fingers, of which nine fingers, suitable for replantation, survived.⁴⁷

Experimental toe-to-hand transplantation was first reported by Buncke et al. in 1966.¹³ Although the human application of this technique was first reported in English literature by Cobbett in 1969,¹⁴ Chen and Yang in 1967 had already performed successful toe-to-hand transfer.^{12,13}

Unlike other organs (such as kidneys or lungs), limb homotransplantation has not been very successful. Canine hind limb homotransplantation is a traditional model because the pre-microsurgical vascular technique (early 1960s or earlier) did not allow the possibility of using small animals.^{48,49} After the perfection of microsurgical technique in the 1960s, rats became an ideal model for studying immune rejection.⁵⁰ Rats can be easily paired according to their histocompatibility and are economical compared to dogs.⁵¹ Furnas et al. were among the first to perform a successful rat leg homotransplantation using an immunosuppressant.⁵² Animal models of limb homotransplantation will continue to play an important role for conquering immunorejection.

B. VASCULARIZED BONE GRAFT OR WHOLE JOINT TRANSPLANTATION

The first vascularized autogenous joint graft was reported by Buncke et al. in 1967.¹⁷ Both the short-term rat knee model and long-term monkey model showed the transplanted joints survived completely with preservation of normal cellular architecture and function. Tamai et al. in 1971 found that a reimplanted canine knee joint showed no degenerative changes even after a year.⁵³ The experimental vascularized rib graft was reported by Strauch et al. in 1971.⁵⁴ Their work was followed by many researchers, leading to extensive use of free vascularized bone grafts in humans for the treatment of bone defects.^{21,22,24}

Vascularized bone or whole joint allograft has been a great challenge since Reeves described the first allograft of a vascularized knee joint in the orthotopic position in dogs using immunosuppression.⁵⁵ Like the case in the whole limb homotransplantation, nothing dramatic will happen without a break-through in immunology.

A creative method, "molded vascularized osteogenesis" was reported by Nettelblad et al. in 1984.⁵⁶ They showed that corticocancellous bone chips placed in a titanium chamber with a vascular pedicle running through it resulted in a suitable size and shape of vascularized bone graft (Figure 7). This concept was supported by Mizumoto et al. who implanted a bone-chip containing polyethylene chamber with saphenous vessels running through it in rabbit thigh muscles.⁵⁷ Recently, two similar models have been reported in which the positive effect of DBM and growth factors (TGF- β and bFGF) on osteogenesis and angiogenesis were found.^{58,59}

C. VASCULARIZED PERIOSTEUM GRAFT

Finley et al. in 1978 reported autogenous periosteum grafts with vascular anastomoses for the repair of bony defect in an animal model.⁶⁰ In 1979, the first clinical use of free periosteal graft for the treatment of congenital pseudoarthrosis of the tibia was successfully conducted in Beijing.⁶¹ Although there has been some concern about the osteogenic properties of vascularized periosteal

grafts,^{62,63} several clinical applications of this technique for repairing bone defects have shown promising results.^{33,64} Recently, researchers have paid attention to periosteum allograft transplantation. Unlike whole limb or whole joint homotransplantation, this new approach seems to work with the help of immunosuppression. The periosteum implanted in muscles formed bone. The one implanted in the radius healed the defect.^{65,66}

D. VASCULARIZED MUSCLE TRANSFER

The first muscle transfer (rectus femoris) in the dog was reported by Tamai in 1968.¹⁶ The transplantation was proven to be successful by electromyography and light and electron microscopy. In 1973, a Shanghai group successfully treated a severe Volkmann's ischemic contracture of the forearm using a free pectoralis major muscle transfer.²⁰ Today, free muscle transfer to restore functional defects in the extremities or to reconstruct facial animation has been well-established.⁶⁷ Rats are economical animals for studying free muscle transfer. Microvascular transfer of gracilis muscles in rats has been reported.⁶⁸

E. REVASCULARIZATION OF AVASCULARIZED BONE

Dickson and Duthie⁶⁹ and Boyd and Ault⁷⁰ implanted arteries into the femoral shaft and head in canine models. They found prolonged blood flow by cineradiographic arteriograms. Hori et al. further studied the effect of vessel transplantation into bone and documented the growth of new blood vessels and new bone formation.⁷¹ Using vascular bundle transplantation, Hori et al. treated nine patients with Kienboeck's disease and one with scaphoid necrosis. The results were promising after a three year follow-up.⁷¹ A rat model of revascularization of ischemic femoral head was also reported.⁷²

Another approach for treatment of osteonecrosis is a vascularized bone graft. Femoral head osteonecrosis has been treated using vascularized fibula graft^{73,74} and vascularized iliac crest graft.⁷⁵ The clinical application was started seven years prior to the first experimental study in a canine femoral head model using vascularized ribs transfer.⁷⁶ Recently, a vascularized periosteal bone graft has been reported to treat osteonecrosis of talus.⁶⁴ Although most cases of femoral head necrosis have been treated with femoral head replacement or total hip arthroplasty, vascularized bone graft still remains as an alternative treatment, especially when the collapse is not severe.^{77,78}

F. VASCULARIZED TENDON GRAFT

There have been two different kinds of vascularized tendon grafts, the composite skin-tendon grafts originally described by Taylor and Townsend,⁷⁹ and the free or pedicle tendon transfer by Morrison.⁸⁰ The former has been often used to treat complicated soft tissue defect involving both tendon(s) and skin. Successful clinical applications have been reported, including the composite grafts of extensor hallucis brevis in dorsal foot flap,⁷⁹ external oblique aponeurosis strips in groin-epigastric flap,⁷⁹ palmaris longus or flexor carpi radialis in Chinese forearm flap,^{81,82} and partial triceps tendon in upper lateral arm flap.⁸³

The free vascularized tendon grafts feature small sizes suitable for reconstruction of flexor tendons in "Zone 2." There is a less likely chance of adhesion, and it promotes gliding due to an intact tendon sheath and vascularity. It also facilitates early healing at repair junctures due to their vascularized nature, and can even improve the vascularity of the tendon bed and the finger.^{80,84} The free vascularized tendon grafts described in the literature include the extensor hallucis longus or brevis, the extensor indicis proprius, the palmaris longus, and the sublimus tendons of the ring and little fingers.^{84,85}

Animal models on this subject were developed after clinical application of the monkey "Zone 2" model by Morrison's group⁸⁶ and the tibialis tendon model in the rabbit by Moriyama.⁸⁷

G. VASCULARIZED NERVE GRAFT

The first free vascularized nerve graft was reported by Taylor and Ham in 1976.⁸⁸ They tested the new technique using a pig femoral neurovascular graft model before applying it to a patient with a median nerve defect. A 24 cm long vascularized superficial radial nerve was grafted into the defect using interfascicular repair technique. Postoperative follow-up showed that the nerve had grown 26 cm distally in six months.

The vascularized nerve grafting has the advantages of (1) early revascularization which may increase the speed of axon and myelin sheath regeneration (average speed is 1 mm per day compared to 1.5 mm per day in Taylor's case) and (2) early revascularization which may decrease the extent of fibroblast infiltration and endoneural scarring. The indication for a vascularized nerve grafting may include a severely compromised recipient bed with a long defect. Usable donor nerves are limited. There have been many reports of donor nerves being used clinically, such as superficial radial nerve,⁸⁸ sural nerve,⁸⁹ ulnar nerve,^{90,91} deep peroneal nerve⁹² and external popliteal sciatic nerve.⁹³

Rabbits are the most commonly used animals for nerve repair, of which vascularized sciatic and median nerve have been reported.⁹⁴⁻⁹⁶ Rat femoral nerves have also been used.⁹⁷

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Appendix 1

Abbreviations

ASTM	American Society for Testing and Materials
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BSE	Backscattered electron microscopy
CSLM	Confocal laser scanning microscopy
CoCr	Cobalt chromium alloy
CSD	Critical size defect
DBM	Demineralized bone matrix
ECM	Extensive extracellular matrix
DEXA	Dual energy X ray absorptiometry
ELISA	Enzyme linked immunosorbent assay
ESAF	Endothelial cell-stimulating angiogenesis factor
FGF	Fibroblast growth factor
GAG	Glycosaminoglycans
GLP	Good laboratory practice
GFs	Growth factors
HA	Hydroxyapatite, hyaluronic acid
IGF	Insulin-like growth factor
IHCS	Immunohistochemical staining
IM	Intramuscularly
IP	Intraperitoneally
ISH	<i>In situ</i> hybridization
KS	Keratan sulfate
MBT	Molecular biological technique
NGF	Nerve growth factor
NSAIDs	Nonsteroidal antiinflammatory drugs
NZW	New Zealand White
OA	Osteoarthritis
OM	Osteomyelitis
ON	Osteonecrosis
OVX	Ovariectomy
PDGF	Platelet-derived growth factor
PGs	Proteoglycans
PGA	Polyglycolic acid
PLA	Polylactic acid
PMMA	Polymethylmethacrylate
QRD	Quantitative roentgenographic densitometry
QUS	Quantitative ultrasound
rhbFGF	Recombinant human basic fibroblast growth factor
RIA	Radioimmunoassay
SC	Subcutaneously

SC tissue	Subcutaneous tissue
SPA	Single-photon absorptiometry
SD rat	Sprague-Dawley rat
TIMP	Tissue inhibitor of metalloproteinase
Tb.N	Number of trabeculae
Tb.Sp	Trabecular spacing or trabecular separation
Tb.Th	Thickness of trabeculae
TGF	Transforming growth factor- β
TJR	Total joint replacement
TCP	Tricalcium phosphate
TBV	Trabecular bone volume
UHMWPE	Ultra high molecular weight polyethylene

Appendix 2

Useful Journals

Acta Orthop. Scand.
Am. J. Vet. Res.
Arch. Orthop. Trauma Surg.
Arthritis Rheum.
Biomaterials
Calcif. Tissue Int.
Calcif. Tissue Res.
Clin. Orthop.
J. Appl. Biomater.
J. Biomech.
J. Biomed. Mater. Res.
J. Bone Joint Surg. (Am)
J. Bone Joint Surg. (Br)
J. Bone Miner. Res.
J. Hand Surg. (Am)
J. Hand Surg. (Br)
J. Invest. Surg.
J. Orthop. Res.
Lab. Anim.
Lab. Anim. Sci.
Orthop. Clin. North Am.
Osteoarthritis Cartilage
Plast. Reconstr. Surg.

Useful Books

Handbook of Biomaterials Evaluation, von Recum, A., Ed., Macmillan, New York, 1986.
Handbook of Laboratory Animal Science, Svendsen, P., Hau, J., Eds., CRC Press, Boca Raton, 1994.



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Appendix 3

Major Sources of Laboratory Animals

Major vendors of laboratory animals include Charles River (www.criver.com), B & K Universal, Taconic (www.taconic.com), and Harlan (www.harlan.com/home.htm). They have branches in Europe, North America, Asia, and other continents. For addresses and phone numbers, go to the internet (search for laboratory animal vendors) or see *Laboratory Animal Buyers' Guide 1996* (along with the 1996 journal issues of *Laboratory Animals*), published for Laboratory Animal Ltd. by PRC Associates, Great Britain.



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